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NATTHAREE CHANCHAREONSOOK

ORAL AND MAXILLO- FACIAL BONE RECONSTRUCTION



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NATTHAREE CHANCHAREONSOOK

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door

Nattharee Chanchareonsook

geboren op 25 april 1974
te Bangkok, Thailand

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ORAL AND MAXILLO-FACIAL BONE RECONSTRUCTION

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CHAPTER 1

INTRODUCTION AND STUDY OBJECTIVES

Nattharee Chanchareonsook

Introduction

Hard and soft tissue reconstruction in the oral and maxillofacial region due to trauma, tumor surgery or congenital deformities remains a challenge for reconstructive surgeons. The current preferred treatment includes the use of vascularized bone grafts, such as pedicled grafts or microvascular free grafts. This even extends to the use of computer-assisted modeling to preoperatively design the tumor resection and contour the microvascular free flap reconstruction by using a customized template. However, the current techniques have inherent disadvantages of donor site morbidity, risk of infection or non-acceptance of the flap as well as a prolonged healing and waiting time before dental rehabilitation can begin.¹

This thesis aims to investigate novel methods of oral and maxillofacial bone reconstruction that may potentially avoid the need to harvest bone from a donor site.

Endoprosthesis

The use of an endoprosthesis for mandibular reconstruction in animal models has been recently described in the literature.²⁻⁶ This device is based on the same principle as the modular endoprosthesis in orthopedic surgery for the reconstruction of long bones.

In orthopedics, the metallic modular endoprostheses for skeletal reconstruction in limb-sparing surgery was first introduced in the mid 1980s. The term “modular” means that the device is prefabricated of different components with various sizes, which can be easily assembled together during surgery. This provides an element of expandability and flexibility and permits a significant reduction in manufacturing costs. Bickels et al., reported in a long term follow-up study that a femoral endoprosthetic reconstruction is a safe and reliable technique of functional limb sparing.⁷ The implant survival rate of custom proximal femoral endoprosthetic replacement has been reported to be as high as 77% at 10 years and 57% at 20 years without revision.⁸

An orthopaedic endoprosthesis can be installed into the remaining healthy bone stump(s) using two different fixation approaches: (1) cemented and (2) cementless. The degree of stress shielding in the bone around a cementless endoprosthesis stem depends on mechanical factors, such as stem stiffness (which may relate to factors such as shape and elastic modulus), pre-operative bone stiffness, interface bonding characteristics, and loading force at the reconstruction site.⁹ On the other hand, the

strength of the stem-cement bond in the case of a cemented stem is relatively low. Once the stem-cement interface becomes debonded, the stresses in the cement can increase dramatically, enhancing the probability of cement-bone interface loosening and, eventually, gross loosening. In addition, the metal may rub against the cement mantle, producing metal and cement particles and increasing the probability of failure due to particulate reactions.

The modular endoprosthesis for mandibular reconstruction was first introduced in oral and maxillofacial surgery in 2008.³ The device is composed of a body part and two stem components, which are assembled together and the stem parts are inserted in the cancellous bone of the remaining bone stump(s). The body of the endoprosthesis functions as replacement for the missing part of mandibular bone. Till now, only a cementation technique has been tested for the fixation of the device in the mandibular stumps. The device stems are fixed with polymethyl methacrylate (PMMA) cement into the remaining mandibular bone stumps. After its introduction, several pre-clinical studies have been done, which focused on the PMMA cement fixation^{10, 11} as well as on biomechanical aspects of the endoprosthesis.¹²⁻¹⁴

No previous studies have been performed to evaluate the feasibility of using a cementless endoprosthesis for mandibular reconstruction. Considering the available knowledge and continuous advancements on the osseointegration of dental implants, it can be hypothesized that a cementless approach can be used for the installation of a mandibular endoprosthesis. For example, dental implants are provided with surface coatings as well as surface roughness in order to increase the bone-implant surface contact. Currently, the mandibular endoprosthesis is in the early stage of development. We are exploring the different possibilities and modifications from the previous knowledge gained from both *in vivo* and *in vitro* studies. Many aspects of this novel device still need to be evaluated, including the design, biomechanics and clinical performance, so that we may have a better understanding. Eventually, we aim to bring its success rate to a level that will allow for clinical use.

Regenerative Medicine

During the last decade, the reconstruction of bone defects has been brought to a next level with the introduction of “*regenerative medicine*”. The term “*regenerative medicine*” was first introduced in 1992, by Dr. Leland Kaiser in a paper on future technologies that will impact hospitals.¹⁵ In 2006, the US National Institutes of

Health defined regenerative medicine as ‘the process of creating living, functional tissues to repair or replace tissue or organ function which has been lost due to age, disease, damage, or congenital defects’. Regenerative medicine intends to support and enhance the physiological mechanisms of tissue repair and regeneration by stimulating the body’s own repair mechanisms to heal previously irreparable tissues or organs and promises to extend healthy life spans and improves the quality of life by supporting and activating the body’s natural healing.¹⁶ This emerging multidisciplinary field, involving biology, medicine, and engineering, is likely to revolutionize the ways to improve the health and quality of life for millions of people worldwide by restoring, maintaining, or enhancing tissue and organ function. Regenerative medicine has recently influenced basic and translational research and is now applied in clinical studies at the translational research stage and makes its way into surgical practice. This holds the promise for custom-tailored constructs with the potential to regenerate tissue in the host without significant donor site morbidity and size limitation.^{17, 18} The ideal reconstructive goals are to return a complete original form and function of the lost tissue. Regenerative medicine is using the principles of “tissue engineering”.^{19, 20}

The paradigm of tissue engineering consists of (1) a **scaffold**, which includes the use of novel biomaterials that are designed to direct the organization, growth, and differentiation of cells in the process of forming functional tissue by providing both physical and chemical cues, (2) **biomolecules**, which includes the application of angiogenic factors, growth factors, differentiation factors and bone morphogenic proteins biomolecules, and (3) **stem cells**, which includes the use of enabling methodologies for the proliferation and differentiation of cells, acquiring the appropriate source of cells such as autologous cells, allogeneic cells, xenogeneic cells, stem cells, genetically engineered cells, and immunological manipulation.

Current investigations evaluating scaffold materials used for bone reconstruction deal with the biomaterials characteristics and their essential properties to favor bone healing. Those properties are based on biocompatibility, osteoconductivity, osteoinductivity, biodegradability, scaffold architecture, surface properties, porosity and permeability and load-bearing or mechanical properties. The ideal scaffold has sufficient strength to protect cells and sufficient porosity to allow nutrient and differentiation factors to diffuse through the scaffold.^{21, 22} However, due to the overall lack of osteoinductive properties in bone scaffolds, biological factors are included to improve the ability to induce bone formation. The scaffold acts as a carrier for biofactors or stem cells for bone defect restoration.

The discovery of bone morphogenetic proteins (BMPs) as osteoinductive agents

and the subsequent development of commercially available recombinant forms of BMPs has offered the potential to replace traditional grafting techniques with *de novo* bone formation.²³ The particular bone morphogenetic proteins (BMP-2 and BMP-7) are often used and have shown promising results for bone regeneration. The quality of the induced and regenerated bone depends on many factors including dosages, ability of the carriers (scaffold) in maintaining and releasing BMP to the surrounding tissue bed.

Further, a combination of bone scaffolds with osteogenic cells has been suggested as a promising strategy to overcome the lack of osteoinductivity.²⁴ Bone marrow mesenchymal stem cells (MSCs) seem to have a promising potential in bone regeneration. Stem cell and stem cell based therapies are related to the ability of the indefinite self-replication of stem cells throughout the life of the organism. Under the right conditions, right signals, stem cells can differentiate into many different cell types including bone cells that regenerate a bone defect.

In bone marrow, a population of progenitor cells is present, which are called mesenchymal stem cells. Purification and culture-expansion of these cells has been shown to result in functional bone regeneration in experimental animals when delivered to the defect site.²⁵

Regenerative Medicine for Mandibular Bone Reconstruction

Regenerative medicine holds also promise for the reconstruction of mandibular bone and can perhaps overcome some of the current surgical limitations. However, the use of a regenerative medicine approach for this application is still in its infancy and only a limited number of studies are available focusing on the use of regenerative medicine for mandibular segmental reconstruction.

This thesis includes an extensive review on tissue engineering approaches for mandibular bone continuity defect reconstruction in Chapter 2. Research is ongoing to improve the material properties of bone scaffolds by enhancing its osteoconductivity, mechanical characteristics and effectiveness as a carrier of biomolecules and stem cells. Various approaches have been explored for mandibular regeneration using a combination of scaffolds with osteogenic cells/tissues and/or bioactive substances. Although tissue engineering approaches to mandibular bone reconstruction demonstrate some clinical potential as an alternative to autogenous bone grafting, problems related to the complex functional biomechanical forces in the mandible and vascularization of the tissue-engineered construct still remain

significant challenges.

Combining the concepts of the modular endoprosthesis and tissue engineering, this thesis also investigates the feasibility of using a modular endoprosthesis made of a bioresorbable scaffold material in combination with growth factors or bone marrow cells for the reconstruction of segmental mandibular defects.

Objectives of This Thesis

The objective of this thesis is to evaluate methods of reconstruction of oral and maxillofacial defects that avoid the harvesting of bone from a donor site, namely: 1) metallic modular endoprosthesis (cementless or non-cemented) for mandibular reconstruction and 2) tissue engineering for alveolar bone reconstruction during immediate dental implant placement (small defect size) and segmental mandibular reconstruction (large defect size).

The thesis therefore seeks to address the following questions:

1. What knowledge is currently available in preclinical *in vivo* as well as clinical literature regarding research methodologies and effectiveness of bone tissue engineering for mandibular continuity defects?
2. Does the use of poly(ϵ -caprolactone) (PCL) implants with or without carbonate-substituted hydroxyapatite (CHA) coating result in a better soft tissue response compared to the commonly used titanium alloy (Ti-6Al-4V)-machined surface?
3. What is the feasibility of using a cementless approach for the installation of a modular mandibular endoprosthesis?
4. Does the use of a mandibular endoprosthetic body component made of poly(ϵ -caprolactone) in combination with rhBMP-2 or autologous bone marrow result in regeneration of the segmental mandibular defect?
5. Can a 3D polycaprolactone-tricalciumphosphate (PCL-TCP) scaffold be used for the regeneration of alveolar ridge defects in combination with immediate dental implant installation?

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TISSUE ENGINEERED MANDIBULAR BONE RECONSTRUCTION FOR CONTINUITY DEFECTS: A SYSTEMATIC APPROACH TO THE LITERATURE

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Introduction

Mandibular continuity defects result from a variety of causes including maxillofacial trauma, osteomyelitis, osteonecrosis and resection of benign or malignant tumors.^{1,2} Unrepaired defects are associated with defacement, reduced masticatory capability and loss of speech, which severely affects the patient's quality of life. Ideally, mandibular continuity defect reconstruction should not only restore the anatomical height and contour of the missing part, but should in addition allow reestablishment of oral function.¹ Until now, autogenous bone transplantation - especially free vascularized tissue transfer - is considered as "gold standard of care" for mandibular reconstruction in patients undergoing major ablative surgery.²⁻⁴ In principle, autogenous bone grafts provide all critical factors for bone regeneration, such as a scaffold for osteo-conduction, growth factors for osteo-induction and cells for osteogenesis.⁵ However, the major problem of this approach is the requirement of autogenous donor tissue which results for example in donor site morbidity.⁶ Moreover, despite the availability of various reconstructive methods by means of autogenous tissue, perfect mandibular reconstruction including restoration of continuity, sensation, dentition, soft tissue, function, as well as aesthetics is still not achievable.^{1,2} As a consequence, mandibular bone reconstruction remains still a challenge.²

However, development of reliable tissue engineering techniques might offer a next step in the evolution of mandibular reconstruction.^{2,7} By definition, tissue engineering was defined as an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function.⁸ Bone tissue engineering is a relatively new method that uses scaffolds, bioactive substances and/or cells/tissues with osteogenic potential.¹ Ideally, the scaffolds should be (1) three-dimensional and highly porous with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste as well as (2) biocompatible and bioresorbable with a controllable degradation and resorption rate to match cell or tissue growth. Furthermore these scaffolds should possess (3) suitable surface chemistry for cell attachment, proliferation, and differentiation, and (4) mechanical properties to match those of the tissues at the site of implantation.⁹ At present, a multitude of scaffolds made of various material¹⁰⁻¹⁹ in combination with bioactive substances or osteogenic bone marrow stromal cells (BMSCs)^{10, 20-26} to initiate or enhance bone formation^{15-17, 19, 27-33} are under study.

In 2006, Ikada defined a concept on methodology in tissue engineering as (1)

placing the construct scaffold in a bioreactor to reconstruct an engineered tissue *in vitro* called *in vitro* (or *ex vivo*) tissue engineering and (2) implantation of the construct scaffold in the body until a new tissue is regenerated *in vivo* called *in vivo* (or *in situ*) tissue engineering.³⁴ However, the construct lacks completely a pre-existing vasculature. Cell survival and tissue formation will depend upon local vasculature and the speed at which a fully functional vascular supply will be developed.³⁵ This makes that the reconstruction of large-volume defects, such as mandibular continuity defects, remains challenging. Therefore, vascularisation concepts gain on interest and the combination of tissue engineering approaches with flap prefabrication techniques. This may eventually allow application of bone-tissue substitutes grown *in vivo* with the advantage of minimal donor site morbidity as compared to conventional vascularized bone grafts.¹² This review included the concepts of tissue engineering using axial vascularisation in engineered bone tissues.

Nonetheless, to the best of the authors' knowledge, animal experiments as well as clinical case reports or studies on the subject of bone tissue engineering for mandibular continuity defects are currently neither systematically reviewed nor synopsized.

Therefore, the purpose of the present report was (1) to review systematically preclinical *in vivo* and clinical literature regarding bone tissue engineering for mandibular continuity defects, and (2) to analyze their effectiveness.

Materials and Methods

Study Design

The scientific, pre-clinical *in vivo* and clinical literature regarding tissue engineered approaches for mandibular bone regeneration in continuity defects (i.e. segmental mandibular defects or total mandibular condyle replacements) was systematically reviewed.

Outcome Variables

In principle, for animal experiments as well as human reports, macroscopical or histological or histomorphometric data on amount of total bone defect bridging were chosen as primary outcome variable. However, for human reports, clinical and/or radiographic evidence of restoration of mandibular continuity were accepted as surrogate outcome variable for the presently defined primary outcome variable. Concomitantly, histological or histomorphometric data of bone ingrowth,

results of biomechanical testing, histological or histomorphometric records of scaffold degradation as well as clinical wound healing were selected as co-outcome variables.

Inclusion/Exclusion Criteria

In general, only animal *in vivo* experiments and human reports presenting macroscopical or histological or histomorphometric data on amount of total bone defect bridging, histological or histomorphometric data of bone ingrowth, results of biomechanical testing, histological or histomorphometric data of scaffold degradation or information related to clinical wound healing as well as human reports presenting clinical and/or radiographic evidence of restoration of mandibular continuity were included.

The following detailed inclusion criteria were used:

1. Research paper presenting *in vivo* animal data;
2. Research paper presenting human data;
3. Defect characteristics should be clearly stated;
4. Implantation site should be clearly mentioned;
5. Reconstructive technique (i.e.: tissue engineering) should be clearly stated;
6. Healing period should be clearly stated;
7. The animal model used should be described conspicuously (species, age)
8. Amount of total bone defect bridging, and/or percentage of bone ingrowths, and/or results of biomechanical testing, and/or percentage scaffold degradation and/or information related to clinical wound healing had to be presented
9. For human reports clinical and/or radiographic evidence of restoration of mandibular continuity had to be presented

Studies that did not meet all above mentioned inclusion criteria, e.g. ex-vivo studies or studies not addressing tissue engineered approaches for mandibular bone regeneration in continuity defects were excluded.

Search Strategy

An electronic search in the database of the National Library of Medicine (<http://www.ncbi.nlm.nih.gov>) up to September 30, 2012, was carried out. Only publications in English were considered and the search was broadened to animals and humans. The following search strategy was applied: ((“tissue engineering”[MeSH Terms] OR (“tissue”[All Fields] AND “engineering”[All Fields]) OR “tissue engineering”[All Fields]) OR (“tissue scaffolds”[MeSH Terms] OR (“tissue”[All Fields] AND

“scaffolds”[All Fields]) OR “tissue scaffolds”[All Fields] OR (“tissue”[All Fields] AND “scaffold”[All Fields]) OR “tissue scaffold”[All Fields]) OR (“reconstructive surgical procedures”[MeSH Terms] OR (“reconstructive”[All Fields] AND “surgical”[All Fields] AND “procedures”[All Fields]) OR “reconstructive surgical procedures”[All Fields] OR “reconstruction”[All Fields]) OR (“bone morphogenetic proteins”[MeSH Terms] OR (“bone”[All Fields] AND “morphogenetic”[All Fields] AND “proteins”[All Fields]) OR “bone morphogenetic proteins”[All Fields] OR (“bone”[All Fields] AND “morphogenetic”[All Fields] AND “protein”[All Fields]) OR “bone morphogenetic protein”[All Fields]) OR (“bone marrow cells”[MeSH Terms] OR (“bone”[All Fields] AND “marrow”[All Fields] AND “cells”[All Fields]) OR “bone marrow cells”[All Fields]) OR (“intercellular signaling peptides and proteins”[MeSH Terms] OR (“intercellular”[All Fields] AND “signaling”[All Fields] AND “peptides”[All Fields] AND “proteins”[All Fields]) OR “intercellular signaling peptides and proteins”[All Fields] OR (“growth”[All Fields] AND “factors”[All Fields]) OR “growth factors”[All Fields])) AND ((“mandible”[MeSH Terms] OR “mandible”[All Fields]) OR ((“mandible”[MeSH Terms] OR “mandible”[All Fields] OR “mandibular”[All Fields]) AND (“Continuity”[Journal] OR “continuity”[All Fields]) AND defect[All Fields]))

Additionally, the ISI Web of Knowledge database was searched operating the same MeSH terms. Again, only publications in English reporting on animal experiments and human studies were considered.

Furthermore, the reference lists of related review articles and publications selected for inclusion in this review were systematically screened.

Study Selection

Two independent reviewers (Nattharee Chanchareonsook [NC] and Leenaporn Jongpaiboonkit [LJ]) initially screened the publication titles and abstracts as identified by the electronic as well as manual search for possible inclusion. Full texts of all papers that were considered eligible for inclusion by one or both of the reviewers were obtained for further assessment against the stated inclusion criteria (Figure 1). Both reviewers used an identical data extraction form to acquire the data independently. Any disagreement between the reviewers regarding inclusion of a certain publication or data extraction was resolved by discussion.

Results

Study Selection

The electronic search in the databases of the National Library of Medicine and ISI Web of Knowledge resulted in the identification of 6727 and 5017 titles, respectively.

As already mentioned, these titles were initially screened by two independent reviewers (NC and LJ) for possible inclusion. In order not to exclude scientific reports unintended, title screening as well as abstract assessment was accomplished to identify articles reporting in general on mandibular defect reconstruction (i.e. non-continuity as well as continuity defects). Title assessment and hand search resulted in final selection of 128 abstracts, 101 full-text articles and 40 scientific papers reporting on tissue engineered reconstruction of mandibular continuity defects that could be included in the present review (Figure 1, Tables 1, 2, 3 and 4). Regarding data extraction and interpretation, any disagreement between the reviewers was resolved by discussion.

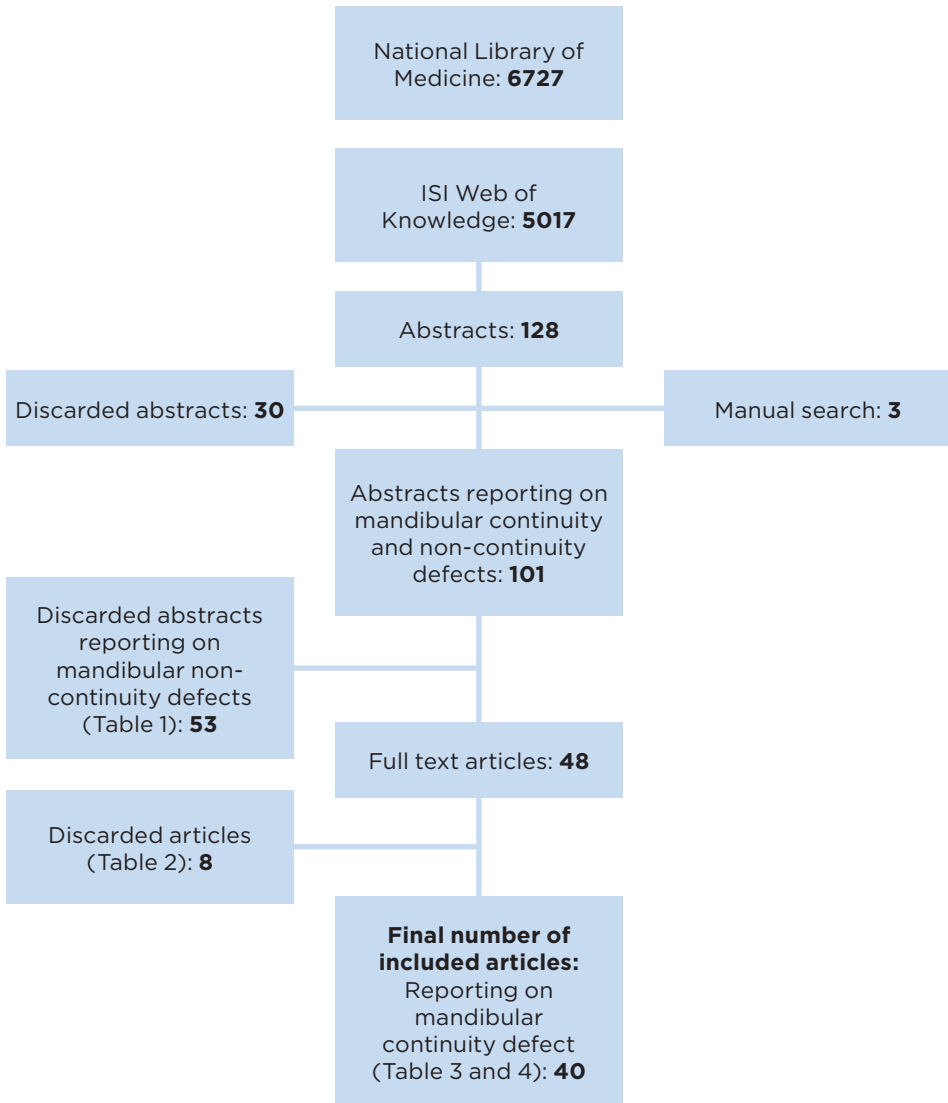


Figure 1. Selection process

Table 1. Excluded articles: Reporting on non-continuity defects

No	Year	Authors	Journal
1.	1998	Schliephake <i>et al.</i> ³⁶	Journal of Oral and Maxillofacial Surgery. 1998;56:616-26.
2.	2002	JP Fisher <i>et al.</i> ³⁷	Engineering in Medicine and Biology. 2002. 24th Annual Conference and the Annual Fall Meeting of the Biomedical Engineering Society EMBS/BMES Conference, 2002. Proceedings of the Second Joint. 2002;1: 827-828.
3.	2002	Chu <i>et al.</i> ³⁸	Biomaterials. 2002;23:1283-93.
4.	2003	Gröger <i>et al.</i> ³⁹	Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery. 2003;37:129-33.
5.	2003	Nakahara <i>et al.</i> ⁴⁰	Tissue Engineering. 2003;9:153-62.
6.	2004	Abukawa <i>et al.</i> ⁴¹	Journal of Oral and Maxillofacial Surgery. 2004;62:601-6.
7.	2004	Yamada <i>et al.</i> ⁴²	Cell Transplantation. 2004;13:343-55.
8.	2004	Yamada <i>et al.</i> ⁴³	Clinical Oral Implants Research. 2004;15:589-97.
9.	2005	Ito <i>et al.</i> ⁴⁴	Journal of Biomedical Materials Research. Part A.2005;73:63-72.
10.	2005	Li <i>et al.</i> ⁴⁵	Australian and New Zealand Journal of Surgery. 2005;75:1017-21.
11.	2005	Marei <i>et al.</i> ⁴⁶	Tissue Engineering. 2005;11:751-67.

12.	2005	Meyer <i>et al.</i> ⁴⁷	The International Journal of Oral & Maxillofacial Implants. 2005;20:882-90.
13.	2005	Ren <i>et al.</i> ⁴⁸	Journal of Biomedical Materials Research. Part A. 2005;74:562-9.
14.	2006	Ito <i>et al.</i> ⁴⁹	Clinical Oral Implants Research. 2006;17:579-86.
15.	2007	Mylonas <i>et al.</i> ⁵⁰	Journal of Prosthodontics. 2007;16:421-30.
16.	2007	Ren <i>et al.</i> ⁵¹	Journal of Biomaterials Science. Polymer Edition. 2007;18:505-17.
17.	2007	Rai <i>et al.</i> ⁵²	Journal of Oral and Maxillofacial Surgery. 2007;65:2195-205.
18.	2007	Wang <i>et al.</i> ⁵³	Biomaterials. 2007;28:3338-48.
19.	2007	Zhang <i>et al.</i> ⁵⁴	Biomaterials. 2007;28:4635-42.
20.	2008	Kuznetsov <i>et al.</i> ⁵⁵	Biomaterials. 2008;29:4211-6.
21.	2008	Tang <i>et al.</i> ⁵⁶	Cell Biology International. 2008;32:1150-7.
22.	2008	Wang <i>et al.</i> ⁵⁷	Journal of Clinical Rehabilitative Tissue Engineering Research. 2008;12: 9762-65
23.	2009	Abukawa <i>et al.</i> ⁵⁸	Journal of Oral and Maxillofacial Surgery. 2009;67:335-47.
24.	2009	Appleford <i>et al.</i> ⁵⁹	Journal of Biomedical Materials Research. Part A. 2009;89:1019-27.
25.	2009	d'Aquino <i>et al.</i> ⁶⁰	European Cells & Materials. 2009;18:75-83.
26.	2009	Guo <i>et al.</i> ⁶¹	Acta Biomaterialia. 2009;5:268-78.

No	Year	Authors	Journal
27.	2009	Jiang <i>et al.</i> ⁶²	Biomaterials. 2009;30:4522-32.
28.	2009	Schliephake <i>et al.</i> ⁶³	International Journal of Oral and Maxillofacial Surgery. 2009;38:166-72.
29.	2009	Schuckert <i>et al.</i> ⁶⁴	Tissue Engineering. Part A. 2009;15:493-9.
30.	2009	Shi <i>et al.</i> ⁶⁵	Journal of Biomaterials Applications. 2009;23:331-46.
31.	2009	Wang <i>et al.</i> ⁶⁶	Biomaterials. 2009;30:2489-98.
32.	2009	Yao <i>et al.</i> ⁶⁷	Journal of Biomedical Materials Research. Part B, Applied Biomaterials. 2009;91:805-12.
33.	2009	Yoshimi <i>et al.</i> ⁶⁸	The Journal of Craniofacial Surgery. 2009;20:1523-30.
34.	2009	Zhang <i>et al.</i> ⁶⁹	Biomedical Materials. 2009;4:045007.
35.	2009	Zhang <i>et al.</i> ⁷⁰	Journal of Controlled Release. 2009;136:172-8.
36.	2009	Zhao <i>et al.</i> ⁷¹	Bone. 2009;45:517-27.
37.	2009	Zheng <i>et al.</i> ⁷²	Journal of Dental Research. 2009;88:249-54.
38.	2010	Gallego <i>et al.</i> ⁷³	Tissue Engineering. Part A. 2010;16:1179-87.
39.	2010	Huang <i>et al.</i> ⁷⁴	Journal of Biomedical Materials Research. Part A. 2010;95:993-1003.

40.	2010	Li <i>et al.</i> ⁷⁵	Journal of Biomedical Materials Research. Part A. 2010:95:973-81.
41.	2010	Parrilla <i>et al.</i> ⁷⁶	Archives of Otolaryngology--Head & Neck Surgery. 2011;137:463-70.
42.	2010	Ribeiro <i>et al.</i> ⁷⁷	Journal of Clinical Periodontology. 2010;37:1128-36.
43.	2010	Zhao <i>et al.</i> ⁷⁸	Oral Diseases. 2010;16:46-54.
44.	2011	Dormer <i>et al.</i> ⁷⁹	Journal of Oral and Maxillofacial Surgery. 2011;69:e50-7.
45.	2011	Kohgo <i>et al.</i> ⁸⁰	The International Journal of Periodontics & Restorative Dentistry. 2011;31:e9-16.
46.	2011	Ito <i>et al.</i> ⁸¹	The International Journal of Oral & Maxillofacial Implants. 2011;26:947-54.
47.	2011	Parrilla <i>et al.</i> ⁸²	Head and Neck. 2010;32:310-8.
48.	2011	Yamada <i>et al.</i> ⁸³	Cell Transplantation. 2011;20:1003-13.
49.	2011	Zhu <i>et al.</i> ⁸⁴	Osteoarthritis and Cartilage. 2011;19:743-50.
50.	2012	Yeo <i>et al.</i> ⁸⁵	Clinical Oral Implants Research. 2012;23:1322-32.
51.	2012	Vahabi <i>et al.</i> ⁸⁶	Chang Gung Medical Journal. 2012;35:28-37.
52.	2012	Zhou <i>et al.</i> ⁸⁷	Materials Science and Engineering: C. 2012;32:994-1000.
53.	2012	Zou <i>et al.</i> ⁸⁸	Public Library of Science one. 2012;7:e32355.

Table 2. Excluded articles: Reasons others than reporting on non-continuity defects

No	Year	Authors	Titles	Journal	Reason(s) of Excluding Articles
1.	1997	Schliephake and Langner ⁸⁹	Reconstruction of the mandible by prefabricated autogenous bone grafts - An experimental study in minipigs.	International Journal of Oral and Maxillofacial Surgery. 1997;26:244-52.	Defect characteristics not clearly indicated
2.	2001	Terheyden <i>et al.</i> ⁹⁰	Mandibular reconstruction with a prefabricated vascularized bone graft using recombinant human osteogenic protein-1: an experimental study in miniature pigs. Part I: Prefabrication.	International Journal of Oral and Maxillofacial Surgery. 2001;30:373-9.	No mandibular reconstruction
3.	2003	Abu-Serriah <i>et al.</i> ⁹¹	The role of ultrasound in monitoring reconstruction of mandibular continuity defects using osteogenic protein- 1 (hOP-1).	International Journal of Oral and Maxillofacial Surgery. 2003;32:619-627	Supplement report of Abu-Serriah <i>et al.</i> /19
4.	2003	Marei <i>et al.</i> ⁹²	Fabrication of polymer root form scaffolds to be utilized for alveolar bone regeneration.	Tissue Engineering. 2003;9:713-31	No mandibular reconstruction

5.	2008	Young <i>et al.</i> ⁹³	Development and characterization of a rabbit alveolar bone nonhealing defect model.	Journal of Biomedical Materials Research. Part A. 2008;86:182-94.	No mandibular reconstruction
6.	2008	Ko <i>et al.</i> ⁹⁴	<i>In vitro</i> osteogenic differentiation of human mesenchymal stem cells and in vivo bone formation in composite nanofiber meshes.	Tissue Engineering. Part A. 2008;14:2105-19.	No mandibular reconstruction
7.	2010	Glied and Kraut. ⁹⁵	Off-label use of rhBMP-2 for reconstruction of critical-sized mandibular defects.	The New York State Dental Journal. 2010;76:32-5.	Reconstruction technique not clearly mentioned
8.	2011	Matsushima <i>et al.</i> ⁹⁶	The nature and role of periosteum in bone and cartilage regeneration.	Cells Tissues Organs. 2011;194:320-5.	No mandibular reconstruction

Table 3. Characteristics of the included articles reporting on continuity defects in *animal experiments*

No	Year	Authors	Species	Defect model	Carrier	Osteogenic cells or tissues BMPs → [total dosage]	Healing period	Considered Outcome Variables
1.	1991	Toriumi <i>et al.</i> ⁹⁷	Dog	Body	Inactive dog bone matrix	rhBMP-2 [0.25 mg]	3 months and 6 months	<ul style="list-style-type: none">• clinical wound healing• bone bridging• bone ingrowths• biomechanics• scaffold degradation
2.	1996	Boyne ²⁹	Monkey	Body	Collagen	rhBMP-2 [not mentioned]	5 months	<ul style="list-style-type: none">• clinical wound healing• bone bridging• bone ingrowths
3.	1999	Toriumi <i>et al.</i> ⁹⁸	Dog	Body	poly(lactide-co-glycolide)	rhBMP-2[not mentioned]	3 months and 30 months	<ul style="list-style-type: none">• clinical wound healing• bone bridging• bone ingrowths
4.	1999	Boyne <i>et al.</i> ⁹⁹	Monkey	Body	collagen sponge	rhBMP-2 [not mentioned]	5 months and 16 months	<ul style="list-style-type: none">• clinical wound healing• bone bridging• bone ingrowths

5.	2001	Schliephake <i>et al.</i> ¹⁰⁰	Sheep	Body	Pyrolyzed bovine bone	Autogenous osteoprogenitor cells	5 months	<ul style="list-style-type: none"> clinical wound healing bone ingrowths
6.	2001	Boyne ¹⁰¹	Monkey	Body	Collagen	rhBMP-2 [not mentioned]	5 months and 6 months	<ul style="list-style-type: none"> clinical wound healing bone bridging
7.	2001	Seto <i>et al.</i> ¹⁰²	Monkey	Body	Poly-D,L-lactic coglycolic acid	rhBMP-2 [3.0, 2.5, or 1.0 mg]	16 weeks	<ul style="list-style-type: none"> clinical wound healing bone bridging
8.	2002	Seto <i>et al.</i> ³¹	Dog	Body	Poly-D,L-lactic coglycolic acid-coated gelatin sponge	rhBMP-2 [not mentioned]	0, 4, 12, 24 and 48 weeks	<ul style="list-style-type: none"> clinical wound healing bone bridging
9.	2002	Marukawa <i>et al.</i> ¹⁰³	Monkey	Body	Poly-D,L-lactic coglycolic acid-coated gelatin sponge	rhBMP-2 [9mg]	15 weeks and 30 weeks	<ul style="list-style-type: none"> clinical wound healing bone bridging
10.	2004	Kontaxis <i>et al.</i> ¹⁶	Sheep	Body	Collagen	rhBMP-7 [not mentioned]	3 months	<ul style="list-style-type: none"> bone bridging biomechanics
11.	2004	Wang <i>et al.</i> ³³	Minipigs	Body	Carboxymethylcellulose stabilized collagenous matrix	rhBMP-7 [3mg]	12 weeks	<ul style="list-style-type: none"> clinical wound healing bone bridging bone ingrowths biomechanics scaffold degradation

No	Year	Authors	Species	Defect model	Carrier	Osteogenic cells or tissues BMPs → [total dosage]	Healing period	Considered Outcome Variables
12.	2005	Fennis <i>et al.</i> ¹⁰⁴	Goat	Angle	Autogenous particulate bone graft mixed with platelet-rich plasma (PRP)	Poly-(D,L-lactide) tray	6 weeks	<ul style="list-style-type: none">• bone bridging• scaffold degradation
13.	2005	Abu-Serriah <i>et al.</i> ¹⁹	Sheep	Body	Collagen	rhBMP-7 [≈ 7mg]	3 months	<ul style="list-style-type: none">• bone bridging• bone ingrowths• biomechanics
14.	2006	Xi <i>et al.</i> ¹⁰⁵	Goat	Body	Natural coral granules	rhBMP-2 [not mentioned] induced autogenous BMSCs	16 weeks	<ul style="list-style-type: none">• clinical wound healing• bone bridging• bone ingrowths• scaffold degradation
15.	2006	Wu <i>et al.</i> ¹⁰⁶	Dog	Body	β-TCP	rhBMP-2 [2.1mg] induced autogenous BMSCs	12 weeks	<ul style="list-style-type: none">• clinical wound healing• bone bridging• bone ingrowths
16.	2006	Seto <i>et al.</i> ¹⁸	Monkey	Body	Collagen beads Collagen sponge	rhBMP-2[2mg] and induced BMCs	24 weeks	<ul style="list-style-type: none">• clinical wound healing• bone bridging• bone ingrowths

17.	2007	He <i>et al.</i> ²⁵	Dog	Body	β -TCP	Osteogenic induced autogenous BMSCs	3 months	<ul style="list-style-type: none"> clinical wound healing bone ingrowths biomechanics
18.	2007	Yuan <i>et al.</i> ⁴	Dog	Body	β -TCP	Osteogenic induced autogenous BMSCs	32 weeks	<ul style="list-style-type: none"> clinical wound healing bone bridging biomechanics
19.	2007	Ayoub <i>et al.</i> ¹⁷	Sheep	Body	Collagen	rhBMP-7 [3.5mg] (applied in muscle for prefabrication)	3 months	<ul style="list-style-type: none"> clinical wound healing bone bridging bone ingrowths
20.	2007	Spector <i>et al.</i> ³²	Dog	Body	Collagen sponge containing HA/TCP granules (compression resistant matrix)	rhBMP-2 [2mg]	26 months	<ul style="list-style-type: none"> clinical wound healing bone bridging bone ingrowths
21.	2009	Nolff <i>et al.</i> ¹⁰⁷	Sheep	Body	β -TCP	Autogenous bone marrow and cancellous bone	12 weeks	<ul style="list-style-type: none"> clinical wound healing bone bridging bone ingrowths scaffold degradation

No	Year	Authors	Species	Defect model	Carrier	Osteogenic cells or tissues BMPs → [total dosage]	Healing period	Considered Outcome Variables
22.	2010	El-Bialy <i>et al.</i> ¹⁰⁸	Rabbit	Condyle	collagen sponge inserted into biodegradable urinary bladder extracellular matrix	Autogenous, osteogenic and chondrogenic differentiated BMSCs	4 weeks	<ul style="list-style-type: none">• bone ingrowths
23.	2010	Nolff <i>et al.</i> ¹¹	Sheep	Body	β-TCP	Autogenous bone marrow and morselized cancellous bone	12 weeks	<ul style="list-style-type: none">• clinical wound healing• bone bridging scaffold degradation
24.	2010	Jégoux <i>et al.</i> ²⁶	Dog	Body	Biphasic calcium phosphate ceramic (HA/β-TCP) - wrapped in a resorbable porcine collagen membrane	Autogenous bone marrow	24 weeks	<ul style="list-style-type: none">• clinical wound healing• bone bridging• bone ingrowths scaffold degradation
25.	2010	Yuan <i>et al.</i> ¹⁰	Dog	Body	Natural coral	Osteogenic induced autogenous BMSCs	12weeks and 32 weeks	<ul style="list-style-type: none">• clinical wound healing• bone bridging• bone ingrowths• biomechanics• scaffold degradation

26.	2010	Zhou <i>et al.</i> ¹⁰⁹	Monkey	Body	Demineralized freeze-dried bone allograft or Coralline hydroxyapatite	rhBMP-2 [4.5mg] (applied in muscle for prefabrication)	26 weeks	<ul style="list-style-type: none"> • clinical wound healing • bone bridging • bone ingrowths • scaffold degradation
27.	2012	Busuttill Naudi <i>et al.</i> ³⁰	Rabbit	Body	β -TCP	rhBMP-7 [400ng]	3 months	<ul style="list-style-type: none"> • clinical wound healing • bone bridging • bone ingrowths • scaffold degradation • biomechanics
28.	2012	Hussein <i>et al.</i> ¹¹⁰	Dog	Body	Collagen sponge	rhBMP-2 [1.6mg]	12 weeks	<ul style="list-style-type: none"> • bone bridging
29.	2012	Herford <i>et al.</i> ¹⁵	Monkey	Body	Collagen sponge or Collagen sponge combined with HA/TCP or ceramic/collagen composite with HA/ TCP (compression- resistant matrix)	rhBMP-2 [0, 6, 12 or 16 mg]	6 months	<ul style="list-style-type: none"> • bone bridging • scaffold degradation

Table 4. Characteristics of the included articles reporting on continuity defects in *human studies*

No	Year	Authors	Number of Patients	Carrier	Osteogenic tissues / BMPs [total dosage]
1.	2001	Moghadam <i>et al.</i> ¹¹¹	1	Allogenic demineralized bone matrix gel	Native human BMPs [200mg]
2.	2002	Ferretti and Ripamonti ¹¹²	6	Allogenic bone matrix	Xenogeneic BMPs [2mg – 8mg]
3.	2004	Warnke <i>et al.</i> ¹¹³	1	Xenogeneic bone mineral blocks	Autologous bone marrow / rhBMP-7 [7mg]
4.	2006	Warnke <i>et al.</i> ¹¹⁴	Supplement report of Warnke <i>et al.</i> ¹¹³		
5.	2007	Herfort <i>et al.</i> ¹¹⁵	2	Collagen sponge	Autogenous bone / rhBMP-2 [8mg] or rhBMP-2 [8mg]
6.	2008	Clokje and Sandoz ¹¹⁶	10	Allogenic demineralized bone matrix in a reverse-phase medium	rhBMP-7 [not mentioned]

7.	2008	Carter <i>et al.</i> ¹¹⁷	4	Collagen sponge or Collagen sponge / allogenic bone	Autogenous bone marrow / rhBMP-2 [12mg] or rhBMP-2 [8.4mg]
8.	2008	Herford and Boyne ¹¹⁸	14	Collagen sponge	rhBMP-2 [4.2mg - 6mg]
9.	2010	Kokemueller <i>et al.</i> ¹¹⁹	1	β -TCP	Autogenous bone / autogenous marrow
10.	2010	Herford and Ciccù ¹²⁰	1	Collagen sponge	Autogenous bone / rhBMP-2 [4.2mg]
11.	2012	Ciccù <i>et al.</i> ¹²¹	1	Collagen sponge / allogenic bone	rhBMP-2 [not mentioned]

General Characteristics of the Included Studies

In total, twenty-nine papers reported on animal experiments. Twenty-seven of these articles presented data on segmental mandibular body reconstruction^{4, 10, 11, 15-19, 25, 26, 29-33, 97-103, 105-107, 109, 110}, one article reported on mandibular angle reconstruction¹⁰⁴, and another one presented data on mandibular condyle reconstruction¹⁰⁸. Research was done in rabbits^{30, 108}, sheep^{11, 16, 17, 19, 100, 107}, goats^{104, 105}, as well as in minipigs³³, dogs^{4, 10, 25, 26, 31, 32, 97, 98, 106, 110} and monkeys^{15, 18, 29, 99, 101-103, 109}. In several studies, teeth were extracted in advance and oral mucosa was allowed to heal completely before resective surgery and reconstructive therapy^{4, 26, 105, 106}. Beside the diversity in animal models, study design as well as healing periods after reconstructive surgery (range: 4 - 48 weeks) were not uniform. The follow up periods were related to difference animal species; e.g. dog (12-48 weeks), monkey (16-30 weeks), sheep (12-20 weeks), rabbit (12-16 weeks), goat (6-16 weeks) and minipigs (16 weeks).

Furthermore, eleven out of the 40 articles presented human data on mandibular reconstruction.¹¹¹⁻¹²¹

The general characteristics of the included animal and clinical studies are summarized in Table 3 and 4.

Animal Studies

Autogenous Bone Precursor Cells or Autogenous Osteogenic Tissues

As described, bone tissue engineering is an approach that combines scaffolds with osteogenic cells/tissues and/or bioactive substances. In preclinical animal models, in principle two different strategies for bone reconstruction in continuity defects have been intensively investigated, i.e. the implantation of autogenous bone precursor cells or autogenous osteogenic tissues - which contain osteoprogenitor cells and/or mesenchymal stem cells - and the application of bone morphogenetic proteins. Both combined with a range of different carrier biomaterials.

In total, 12 scientific papers^{4, 10, 11, 18, 25, 26, 100, 104-108} reporting on ***autogenous bone precursor cells or autogenous osteogenic tissues*** were finally appropriate for inclusion in the current systematic review. Due to their experimental diversity these studies are briefly summarized. (*Details can be found in journal online: Addendum No. 1*)

In summary, ***autogenous bone precursor cells or autogenous osteogenic tissues*** were primarily combined with calcium phosphate ceramic scaffolds, such as β -TCP^{4, 11, 25, 26, 106} and biphasic calcium phosphate ceramic²⁶, or pyrolyzed bovine bone100 or calcium carbonate, such as natural corals^{10,105}. Considering the primary outcome variable bone bridging^{4, 11, 18, 26, 104-107, 117} as well as the co-outcome variables bone ingrowth¹⁰⁰ and biomechanical testing^{4,10,25}, autogenous bone precursor cells or autogenous tissues seeded onto calcium phosphate ceramic scaffolds showed in **preclinical animal studies** the potential of an alternative to autograft bone for mandibular bone reconstruction in continuity defects. Moreover, autogenous bone precursor cells or autogenous osteogenic tissues seeded onto or mixed with collagen sponges^{18,108} demonstrated in **preclinical animal studies** promising results in terms of the primary outcome variable bone bridging¹⁸ or the co-outcome variable bone ingrowths¹⁰⁸. In contrast, autogenous bone precursor cells containing tissues filled in preshaped poly – D, L –lactide trays did not show such a potential as an alternative to autograft bone for mandibular bone reconstruction¹⁰⁴.

Bone Morphogenetic Proteins

Furthermore, in total 15 scientific papers^{15, 18, 29, 31, 32, 97-99, 101-103, 105, 106, 109, 110} reporting on ***recombinant human morphogenetic protein-2*** (rhBMP-2) as well as 5 publications^{16, 17, 19, 30, 33} presenting data on ***recombinant human morphogenetic protein-7*** (rhBMP-7) were eventually included in the current systematic approach. Thereby, the in brief summarized studies of Kontaxis *et al.*¹⁶, Abu-Serriah *et al.*¹⁹, Boyne^{29, 101}, Busuttill Naudi *et al.*³⁰, and Toriumi *et al.*⁹⁷ may give a good general impression of the effectiveness of rhBMPs regarding quantity as well as quality of induced bone for the reconstruction of continuity mandibular defects. (*Details can be found in journal online: Addendum No. 2*)

In summary, ***rhBMP-2*** and ***rhBMP-7*** were studied in combination with collagen/ collagen composite scaffolds^{15-19, 29, 32, 33, 99, 101, 108, 110}, poly-D,L-lactide coglycolic acid based carriers^{31, 98, 102, 103}, β -TCP³⁰ as well as coralline hydroxyapatite¹⁰⁹ and allogenic bone matrix⁹⁷. Regarding the primary outcome variable bone bridging^{15-19, 29-33, 97-99, 101-103, 109, 110} as well as the co-outcome variables bone ingrowths^{17-19, 29, 30, 32, 33, 97-99, 109} and biomechanical testing^{16, 19, 30, 33, 97}, rhBMP-2 and rhBMP-7 demonstrated in **preclinical animal studies** their potential as an alternative to autograft bone for mandibular bone reconstruction in continuity defects. However, the published results were not uniform. It should be mentioned that in different

reports rhBMP-2 or rhBMP-7 combined with demineralized freeze-dried bone allograft¹⁰⁹, polyglycolic co-lactic acid¹⁰² as well as a bovine collagen type I carrier wrapped into a sterno-occipitalis muscle flap¹⁷ were not associated with predictable defect bridging.

In line with these results are the reported effects of rhBMP-2^{15,31,32,98,99,103,110} and rhBMP-7³³ for mandibular bone regeneration in continuity defects. On the other hand, it should be mentioned that rhBMP-2, in the reports of Zhou *et al.*¹⁰⁹ and Seto *et al.*¹⁰², as well as rhBMP-7, in the paper of Ayoub *et al.*¹⁷, have not always been associated with bony union. Likewise, in the paper of Ayoub *et al.*¹⁷, rhBMP-7 was not in all animals associated with complete bone regeneration.

Human Case Report

In addition to animal experiments, bone tissue engineering for reconstruction of mandibular continuity defects has been investigated in humans. Similarly to preclinical models, considerable interest for therapeutic use has been focused on the application of autogenous osteogenic tissues or bone morphogenetic proteins, both combined with a range of different carrier biomaterials. In total, 11 scientific papers¹¹¹⁻¹²¹ reporting on 10 different investigation entities were finally included in the current systematic review.

Autogenous osteogenic tissues and Bone morphogenetic proteins (*Details can be found in journal online: Addendum No.3*)

In summary, transplantation of tissue engineered **autogenous osteogenic tissues** without additional application of osteoinductive BMPs¹¹⁹ or in combination with rhBMP-2^{115,117,120} as well as rhBMP-7^{110,111} was associated with restored mandibular continuity in five cases, but in one case¹¹⁷ no bony union was observed. Furthermore, in 16 patients in some reports^{115,118,121}, osteoinductive rhBMP-2 loaded onto different biomaterials without concomitant transplantation of autogenous osteogenic tissue was followed by restored mandibular continuity. Again, in one subject this did not occur¹¹⁷. Moreover, in 10 patients rhBMP-7¹¹⁶, in one patient native human BMPs¹¹¹ and in two patients xenogeneic BMPs¹¹² without concurrent transplantation of autogenous osteogenic tissue were associated with reconstructed mandibular continuity. However, this was not observed in four subjects treated with xenogeneic BMPs¹¹².

Discussion

Currently bone tissue engineering can be considered as a highly promising approach and as an alternative bone source. Well-performed *in vitro* and *in vivo* experiments are essential to determine the suitability of the chosen concept and to understand the risks before proceeding into the clinical trial.^{122, 123} *In vitro* studies require a desired monitored environment that mimics the dynamics of the *in vivo* condition by a controlled homogeneity of nutrients media (also in terms of pH/osmolarity), additional osteogenic stimuli(s) and providing a physical stimulation as relevant key components for bone construction.¹²⁴ However, the *in vitro* condition is unable to provide physiological function and never being exact the same condition as *in vivo*.¹²⁴ The results from *in vitro* studies do not give direct information or can be difficult to infer to the *in vivo* situation¹²⁵, but are rather considered as baseline properties¹²⁶. For this reason, the use of animal models is often an essential step in the testing of tissue engineering prior to clinical use.

The aims of the present report were to review systematically preclinical *in vivo* as well as clinical literature regarding bone tissue engineering for mandibular continuity defects and to analyze the effectiveness of this approach for the treatment of mandibular continuity defects.

In total, 29 publications reporting on animal experiments and 11 papers presenting human cases could be included in the present systematic review. The evaluated articles of the first part of the current review report on tissue engineered reconstructions of segmental mandibular body, angle or condyle defects in different animal species. Thereby, autogenous bone precursor cells or autogenous osteogenic tissues were primarily combined with calcium phosphate ceramic scaffolds. Regarding bone bridging, bone ingrowth as well as biomechanical testing, these tissue engineered approaches demonstrated a certain potential as an alternative to autograft bone for mandibular bone reconstruction in continuity defects. In principle, these results were not unexpected and were in line with the literature for bone tissue engineering in general. It is well known that BMSCs are capable of self-renewal and differentiation into various osteogenic lineage cells¹²⁷. Furthermore, their osteogenic potential has been demonstrated both *in vitro* and *in vivo*. Consequently, BMSCs became a major seed cell source for bone tissue engineering. Moreover, many previous studies have succeeded in repairing bone defects by using BMSCs in animal models as well as in humans.^{24, 127-129}

Besides, due to their compositional similarities to bone mineral, their excellent biocompatibility, osteoconductivity as well as drug delivery potential, calcium

phosphates, especially tricalcium phosphate and hydroxyapatite, are the most widely used bone substitutes in bone tissue engineering¹²⁷. Moreover, BMSCs seeded onto calcium phosphate scaffolds induced ectopic bone formation in a mice model.¹²⁹

However, the currently presented favorable data for bone tissue engineered constructs as compared to scaffolds alone have to be interpreted with caution. In principle, sample-size and thereby statistical power of the reviewed pre-clinical *in vivo* experiments tended to be low. For example, the compared twelve weeks bone bridging and bone ingrowths¹⁰⁶ originate from only two animals/segmental defects without statistical analysis. Another example is the three months data²⁵. Their statistically significant better biomechanical results ($p < 0.05$) for bone tissue engineered bone as compared to the scaffold alone originate from not more than three animals/segmental defects. Thus, with an assumed α -error of 0.05, post hoc analysis for e.g. compression strength reveals a statistical power as low as 0.385. Furthermore, next to autogenous bone precursor cells or autogenous osteogenic tissues, bone morphogenetic proteins (i.e.: *rhBMP-2* and *rhBMP-7*) were studied. Predominantly, *rhBMP-2* and *rhBMP-7* were combined with collagen/collagen composite scaffolds. However, a few papers examined combinations with poly-D,L-lactide coglycolic acid as well as calcium phosphate carriers. Regarding bone bridging, bone ingrowth as well as biomechanical testing, these tissue engineered approaches displayed some potential as an alternative to autograft bone for mandibular bone reconstruction in continuity defects. However, the published results were not uniform. For example, *rhBMP-2* or *rhBMP-7* combined with a bovine collagen type I carrier¹⁷, polyglycolic co-lactic acid¹⁰² as well as demineralized freeze-dried bone allograft¹⁰⁹ were not associated with predictable defect bridging. Overall, the published outcomes for bone morphogenetic proteins were not surprising and were in line with the reports for bone tissue engineering in general. The osteoinductive potential of BMP-2 and BMP-7¹³⁰ as well as the general importance of carrier selection in conjunction with growth factor application^{15, 115, 131-133} are well known. Also for these reviewed pre-clinical *in vivo* experiments, sample-size and thereby transferability tended to be low. For instance, the three months bone bridging data and the found wide range of mechanical properties of Abu-Serriah *et al.*¹⁹ were obtained from not more six animals/segmental defects. Another good example is the publication of Boyne²⁹. Their five months bone bridging and bone histology data originate from only three animals/segmental defects. Unfortunately, a meta-analytical approach to increase the power of statistical analysis by pooling the results of all retrieved available trials was not feasible. Research of results that are combined in a meta-analysis should preferably be done in a similar manner. As shown in Table

3, this is clearly not the case for the presently included papers. The publications, which were eligible for inclusion in the present study, display experimental variability for the utilized animal model, the anatomical site of reconstruction, the used bone tissue engineering approach, the number of enrolled animals/defects as well as the healing time after reconstruction.

Apart from BMPs, alternative growth factors may serve as potential therapeutic agents to enhance bone and cartilage formation e.g. recombinant human platelet-derived growth factor (rhPDGF), transforming growth factor-beta (TGF- β), fibroblast growth factor, recombinant human growth/differentiation factor-5 (rhGDF-5) and insulin-like growth factor.¹³⁴ PDGF is known to simulate angiogenesis through activation of the macrophages¹³⁴, which secrete factors cells to form new capillary sprouts. Transforming growth factor- β 1) has been proven to promote cartilage regeneration.⁷⁹ RhGDF-5 has the potential to grow the same type of tissues as where it is naturally present. Its possible using in a tissue engineering approach has been reported for the regeneration of dento-alveolar tissues.^{135, 136}

However, a single dose of an exogenous protein will not induce adequately a biologic response in compromised tissue conditions. Gene therapy is another concept in which genetic information is transferred into target cells. Subsequently, the cells synthesize the endogenous protein encoded by the gene.¹³⁷ The process that involves the transfer of functional genetic information into the target cell is known as transduction. This is accomplished when the recombinant vector (virus) that contains the therapeutic DNA binds to the cell, usually via a receptor-mediated process, and then enters that cell. The DNA passes into the nucleus of the cell, where it may become integrated into the host genome or may remain extrachromosomal. The transduced cells can then produce and secrete the growth factor encoded by the DNA⁹⁸⁻¹⁰⁰.^{56, 70} In this review, the use of gene therapy was found being applied in the reconstruction of mandibular continuity defects in animals and humans. On the other hand, gene therapy has been reported for the repair of the mandibular condyle and temporomandibular joints and was found to support mineralized tissue formation.¹³⁸⁻¹⁴⁰

In the second part of the review eleven papers, presenting human cases regarding tissue engineered reconstruction of mandibular continuity defects, were eventually included. The review included the reports on microvascular tissue transfer of prefabricated bones in the study. Although, these techniques belong to tissue regeneration, they are important for the reconstructive surgeon. Thereby, transplantation of tissue engineered autogenous osteogenic tissues without additional application of osteoinductive BMPs or in combination with rhBMP-2/rhBMP-7

produced restored mandibular continuity in five out of six cases. Furthermore, in 29 out of 34 patients, the application of native human BMPs, xenogeneic BMPs, rhBMP-2 or rhBMP-7 without concomitant transplantation of autogenous osteogenic tissue was associated with complete bony defect bridging. Unfortunately, no direct comparison of the results with autogenous bone transplantation can be done due to lack of direct control. However Herford and Ciccù¹²⁰ stated that bone growth cytokines can be considered as a predictable alternative to traditional grafting techniques. In general, these results were not astonishing and in line with the literature^{15, 115, 141-144}. Thus, it might be assumed that these tissue engineered approaches may have, in certain selected patients, some potential as an alternative to autograft bone for mandibular bone reconstruction in continuity defects. However, it should be underlined that until now only a few successfully treated cases have been published. Furthermore, to date the clinical predictability has to be questioned. An additional issue is the limited license of the use of rhBMP-2 in oral and maxillofacial surgery. According to the Center for Devices and Radiological Health (CDRH) of the U.S. Food and Drug Administration (FDA), rhBMP-2 is not licensed for use in surgery of mandibular continuity defects and may only be applied for sinus augmentation and localized alveolar ridge augmentation.

Conclusions

The reviews showed a various study methodology, review period and different control groups. Not all studies compared the finding with a reconstruction with autologous bone substitute. None of the human study was performed as a randomized control trial study. Within the limits of this systematic approach to the literature regarding tissue engineered bone reconstruction in continuity defects of the mandible, we conclude that: (1) published preclinical *in vivo* as well as clinical data are limited, and (2) tissue engineered approaches demonstrate some clinical potential as an alternative to autograft bone. The future research in this area needs to include process evaluation research in order to define the characteristics contributing to the success and failure of any intervention.

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Addendum

Addendum No .1

In a **rabbit** unilateral, segmental defect model of the **temporomandibular joint condyle**, El-Bialy *et al.*¹⁰⁸ evaluated tissue-engineered mandibular condyles (TEMCs). TEMCs consisted of osteogenic and chondrogenic differentiated autogenous bone marrow stem cells (BMSCs) seeded into distinct sections of collagen sponges, which were inserted into biodegradable urinary bladder extracellular matrix (UBM ECM) scaffolds. They compared TEMCs combined with low-intensity pulsed ultrasound (LIPUS) (TEMC + LIPUS) versus TEMCs without LIPUS (TEMC) versus empty scaffolds with LIPUS (Scaffold + LIPUS) versus empty scaffolds without LIPUS (Scaffold). After a healing period of 4 weeks, histomorphometric bone trabeculae /osteogenic tissue measurements revealed for TEMC + LIPUS $1.2 \mu\text{m}^2$ (SD: $0.5 \mu\text{m}^2$), for TEMC $1.1 \mu\text{m}^2$ (SD: $0.1 \mu\text{m}^2$), for Scaffold + LIPUS $0.5 \mu\text{m}^2$ (SD: $0.5 \mu\text{m}^2$), for Scaffold $0.3 \mu\text{m}^2$ (SD: $0.1 \mu\text{m}^2$) and for the normal condyles $1.2 \mu\text{m}^2$ (SD: $1.4 \mu\text{m}^2$). The differences between the normal condyles, TEMC + LIPUS and TEMC were not statistically significant. However, there was statistically significant less bone trabeculae/osteogenic tissue formation in the UBM ECM scaffolds with or without LIPUS as compared to normal condyles or TEMC + LIPUS ($p < 0.05$). Data regarding proportion of total bony defect bridging, biomechanical testing, percentage scaffold degradation or clinical wound healing were not presented. Finally, it was concluded that the use of UBM ECM along with chondrogenic and osteogenic differentiated BMSCs showed promise for joint tissue engineering of mandibular articular condyles and that UBM ECM without cells is not a practical option for mandibular condyle tissue engineering.

Fennis *et al.*¹⁰⁴ assessed in a **goat** unilateral, segmental defect model of the **mandibular angle** preshaped poly – D,L –lactide (PDLA) trays filled with an autogenous particulate bone graft taken from the anterior iliac crest mixed with platelet-rich-plasma (PRP). It was hypothesized that these grafts would heal, providing bone continuity with the grafted bone. After six weeks of healing, in all cases macroscopically the defects were bridged by newly formed bone on the buccal and lingual aspect of the mandible. However, evaluation of cross-sections showed that the PDLA scaffolds had shrunk, thus narrowing the space containing the grafted bone. Furthermore, light microscopic evaluation showed that extensive resorption of the particulate bone grafts had taken place. The remaining particulate bone chips within the confines of the PDLA tray showed signs of remodeling, predominantly osteoclastic activity as well as some deposition of osteoid tissue. Moreover, the PDLA scaffolds itself showed no signs of degradation and in all specimens the PDLA scaffolds were encapsulated by fibrous tissue. However, on the external surface of the PDLA scaffold, callus formation provided bone continuity between the mandibular stumps. In one animal, with loose screws of the osteosynthesis plates, a necrotic grafted area without any signs of remodeling directly adjacent to the PDLA scaffold was seen. Nonetheless, also in this specimen abundant callus formation had appeared, providing bony continuity of both mandibular stumps. It should be mentioned that this experiment was uncontrolled and data regarding biomechanical testing were not presented. Finally, it was concluded that the study did not completely confirm the formulated hypothesis of healing by bone continuity with the grafted bone. However, because bony continuity between the mandibular stumps was provided by newly formed bone on the outside of the scaffold, maintaining the original shape of the reconstructed area, it was supposed that PDLA scaffolds might be a promising alternative in mandibular reconstruction.

Moreover, Xi *et al.*¹⁰⁵ studied in a **goat** unilateral, segmental defect model of the **mandibular body** recombinant human morphogenetic protein-2 (rhBMP-2) induced autogenous marrow mesenchymal stem cells (MSCs) seeded into natural coral granules (test) against unloaded natural coral granules (control), both implanted into a titanium reticulum stent. After 16 weeks of healing, macroscopically the graft surfaces of the test group were covered with smooth and glistening red bone tissue. Furthermore, the shape of the newly formed bone grafts was coincident with that of the original host bone. In contrast, in the control group, coral scaffolds were absorbed and had rough and white surfaces. Histologically, new bone formation was demonstrated in all specimens of the test group, whereas no

evidence for osteogenesis in the center of the titanium reticulum was found in the control group. In spite of this, also in the control group bone healing was observed outside the titanium reticulum and explained by periosteal osteogenesis. In addition, this explains the clinical finding that survived animals in the experimental as well as in the control group recovered mandibular continuity. However, quantitative data on proportion of total bony defect bridging, bone ingrowths, scaffold degradation or results of biomechanical testing were not presented. Finally, the potential of rhBMP-2 induced MSCs seeded into natural coral granules for mandibular continuity defect reconstruction was assumed.

Furthermore, in a **sheep** unilateral, segmental defect model of the **mandibular body**, Schliephake *et al.*¹⁰⁰ evaluated autogenous osteoprogenitor cells cultivated from bone biopsies from the iliac crest and seeded into scaffolds of pyrolyzed bovine bone (test) against the empty scaffold (control). After five months of healing, in the test group on average 34.4% of the evaluated surface units exhibited bone formation. However, the distribution of newly formed bone across the defect was variable. The marginal sections of the defect exhibited bone tissue on average in 43.5% and 46.5% of the evaluated surface units. These values decreased in the intermediate and central parts to 19.7%, 23.8% and 20.3%, respectively. Furthermore, bone quantity of the five sections was statistically significant different ($p < 0.05$). In contrast, in the control group exhibited on average 10.4% of surface units bone formation. Once more, the distribution of newly formed bone across the defect was variable. The marginal sections had the highest percentage of bone fill with 19.5% and 22.4% of the evaluated units. The intermediate and central parts showed very low values with the presence of bone tissue in only 0.2%, 3.0% and 3.7% of the evaluated units. Also the distribution of the newly formed bone in the five sections of the control group was statistically significant different ($p < 0.05$). Furthermore, the difference in extend of bone formation between test and control group was statistically significant different ($p < 0.05$). Data regarding proportion of total bony defect bridging, biomechanical testing, percentage scaffold degradation or clinical wound healing were not presented. Finally, it was concluded that extend of bone regeneration in calcium phosphate scaffolds in segmental defects of the sheep mandible can be enhanced by the presence of cultivated autogenous osteoprogenitor cells.

Also Nolff *et al.*¹⁰⁷ used a **sheep** unilateral, segmental defect model of the **mandibular body** to compare blood soaked, beta-tri calcium phosphate (β -TCP) cylinders loaded with autogenous bone marrow and cancellous bone (test) with blood soaked, β -TCP cylinders (control). After 12 weeks of healing, in the test

group complete bony union was achieved. Moreover, the main parts of the ceramic scaffolds were degraded and the residual material was integrated within the newly formed bone. On the contrary, in the control group bone ingrowths into the defect was rare and none of the specimens achieved defect union. Furthermore, incomplete osseointegration of the ceramic material that mainly occurred in close proximity to the cut ends of the mandible was observed. Moreover, direct bone-to-scaffold contact was rare due to an intervening layer of fibrous tissue. In addition, the core of the ceramic graft was intact and the void pore spaces were completely filled by highly cellular vascularized fibrous tissue. Accordingly, within the central parts of the scaffolds in the test group statistically significant more bone formation and statistically significant less residual graft material was found ($p < 0.05$). Unfortunately, percentages of total bone area as well as ceramic area were not presented as overall means. Furthermore, two sheep (one of each group) presented total graft failures with inflammation of the graft side associated with sequestration at the time of sacrifice. Moreover, biomechanical testing was not done. Finally, it was proposed that blood soaked, β -TCP cylinders loaded with autogenous bone marrow and cancellous bone may qualify as a promising alternative to autograft bone for mandibular reconstruction.

Furthermore, Nollf *et al.*¹¹ investigated in a **sheep** unilateral, segmental defect model of the **mandibular body** blood soaked, beta-tri calcium phosphate (β -TCP) cylinders loaded with autogenous bone marrow aspirate and morselized cancellous bone received during segment mandibulectomy (test) versus blood soaked, β -TCP cylinders (control). After twelve weeks of healing, in the test group complete bony union was achieved. Moreover, the main parts of the ceramic scaffolds were degraded and the residual material was integrated within the newly formed bone. Three-dimensional reconstruction of the residual cylinders showed extensive mean scaffold degradation of 94.2% (SD: 3.3%) as well as fragmentation of the ceramic material. On the contrary, in the control group incomplete osseointegration of the ceramic material that mainly occurred in close proximity to the cut ends of the mandible was observed. Moreover, the remaining graft surface was enveloped by woven bone with an intervening layer of radiolucent soft tissue while the center of the defect was occupied by moderately degraded ceramic material. In addition, three-dimensional reconstruction of the residual cylinders showed a mean scaffold degradation of 53.6% (SD: 9.7%). Furthermore, two sheep (one of each group) presented total graft failures with inflammation of the graft side associated with graft displacement and sequestration as well as necrosis of the mandibular bone at the time of sacrifice. Data regarding percentage of bone ingrowths or biomechanical

testing were not presented. Finally, it was concluded that ceramic bone –graft substitutes such as β -TCP represent a promising solution for reconstruction of large bone defects.

Likewise, Wu *et al.*¹⁰⁶ assessed in a **dog** unilateral, segmental defect model of the **mandibular body** recombinant human bone morphogenetic protein-2 (rhBMP-2) osteogenically induced autogenous bone marrow stromal cells (BMSCs) seeded on porous β -tricalcium phosphate (β -TCP) versus β -TCP without BMSCs or transplanted autologous iliums. After twelve weeks of healing, the gross resected specimens showed that the mandibles with BMSCs/ β -TCP constructs or autologous bone had repaired defects and restored mandibular continuity. Moreover, in the BMSCs/ β -TCP group, tissue engineered bone was observed to completely bridge the defect and was indistinguishable at the margins from native bone. In contrast, only partial repair was seen in the β -TCP without BMSCs group. In this group, only a thin bone bridge was observed on the top of the defect and no bony tissue was observed in other sections of the defect site. Histological, in the BMSCs/ β -TCP group abundant mature bone and in the autogenous bone group substantial bone formation was found. In contrast, in the β -TCP without BMSCs group large parts of the scaffold degraded with only scattered bone tissue enclosed. Furthermore, in this group the osteoid synthesis was poor compared with the corresponding cell-scaffold transplant. Clinically, no signs of inflammation or adverse tissue reactions around the implants were observed. Data regarding percentage of bone ingrowths, biomechanical testing or percentage scaffold degradation were not presented. Finally, it was concluded that BMSCs/ β -TCP is a vulnerable alternative to autograft in the treatment of traumatic bone defects and atrophic non-unions, by which the disadvantages of the harvest of autologous bone can be prevented.

Also Yuan *et al.*⁴ compared in a **dog** unilateral, segmental defect model of the **mandibular body**, osteogenically induced autogenous bone marrow stromal cells (BMSCs) seeded on porous β -tricalcium phosphate (β -TCP) (test) versus resected autologous mandibular segments or β -TCP without BMSCs. After thirty-two weeks of healing, macroscopically the mandibles repaired with BMSCs/ β -TCP constructs or autologous mandibular segments achieved bony-union. Moreover, the shapes of repaired mandibles were very close to that of normal edentulous mandibles. On the contrary, β -TCP without BMSCs was not associated with bony-union. Furthermore, in the test group bony-union was microscopically observed without clear boundary between newly formed bone and native bone. Also in the autograft group a bony-union, with intrinsic dead bone and newly form bone coexisting in the repaired area, was observed. In contrast, in the β -TCP without BMSCs group,

only fibrous tissues in the defect area with minimal new bone formation at the cutting ends were observed. In addition, to evaluate the biomechanical properties of the repaired mandibles after thirty-two weeks of healing a three-point bending test was performed. In the BMSCs/ β -TCP group, bending strength load was 1.8 kN (SD: 0.4 kN), bending displacement was 1.9 mm (SD: 0.8 mm), bending stress was 44.7 MPa (SD: 10.1 MPa) and Young's modulus was 437.2 MPa (SD: 199.5 MPa). For the contralateral normal edentulous mandible bending strength load was 3.0 kN (SD: 1.1 kN), bending displacement was 3.7 mm (SD: 1.9 mm), bending stress was 54.4 MPa (SD: 4.3 MPa) and Young's modulus was 275.8 MPa (SD: 107.3 MPa). Furthermore, for the autograft group bending strength load was 2.7 kN (SD: 0.3 kN), bending displacement was 2.6 mm (SD: 0.4 mm), bending stress was 43.1 MPa (SD: 12.0 MPa) and Young's modulus was 250.0 MPa (SD: 77.3 MPa). No statistically significant differences, neither between the BMSCs/ β -TCP group and the normal edentulous mandible nor between the BMSCs/ β -TCP group and the autograft group were found. Because there was no bony-union in the β -TCP without BMSCs group, three point bending test could not be applied in this group. Quantitative data on bone ingrowths or scaffold degradation were not presented. The authors concluded that engineered bone from osteogenically induced BMSCs seeded onto biodegradable β -TCP can well repair critical sized segmental mandibular defects in canines.

Furthermore, He *et al.*²⁵ investigated in a **dog** unilateral, segmental defect model of the **mandibular body**, osteogenically induced autogenous bone marrow stromal cells (BMSCs) seeded on biodegradable and porous β -tricalcium phosphate (β -TCP) (test) versus β -TCP without BMSCs (control). After three months of healing, histologic micrographs demonstrated new bone formation together with osteoblast seems, osteoclastic resorption and cartilage formation in central sections of the scaffolds in the test group. However, control group data were not presented. Additionally, to appraise biomechanical properties of the test and control treatment, compression strength, stress and strain were assessed. On average, in the test group compression strength was found to be 102.8 N, stress 3.5 N/mm² and strain 17 %, whereas in the control group compression strength was found to be 42.9 N, stress 1.9 N/mm² and strain 54.5 %. All differences were statistically significant ($p < 0.05$) and might be in part explained by a significant difference of the mean height of the reconstructed mandible (test: 18.5 mm, control: 9.2 mm, $p < 0.05$). Moreover, gross macroscopical inspection as well as 3D computed tomographic imaging of the test mandibles showed no obvious absorption, whereas apparent absorption was found in the control group. However, qualitative or quantitative data on bone

bridging, quantitative data on bone ingrowths or histomorphometric data on scaffold degradation were not provided. The authors presume the clinical potential of osteogenically induced autogenous bone marrow stromal cells (BMSCs) seeded onto biodegradable and porous β -tricalcium phosphate (β -TCP) in segmental mandibular defect reconstruction.

Moreover, Jégoux *et al.*²⁶ examined in a **dog** unilateral, segmental defect model of the **mandibular body**, delayed autologous bone marrow grafts injected into macropores biphasic calcium phosphate ceramic (MBCP) wrapped in a cross-linked porcine collagen membrane (test) versus delayed autologous bone marrow grafts injected into empty not reconstructed defects (control). Twenty-four weeks after mandibular segmental resection along with reconstruction and sixteen weeks after autologous bone marrow graft injection, complete defect bridging was seen in all test sites, whereas nonunion was observed in all control defects. However, these data were retrieved by three-dimensional reconstruction from x-ray microtomographs and not by histology. Moreover, new bone formation both surrounding the MBCP granules and within the macropores of the granules was verified histological. Nonetheless, it should be mentioned that spontaneous mucosal fistulas in regard to the bone defect occurred at the seventh postoperative day in all animals (test and control). For that reason, every animal underwent a further surgical procedure that finally leads to adequate mucosal healing. However, quantitative data of bone ingrowths, results of biomechanical testing or data regarding scaffold degradation were not given. Finally, the authors concluded that the composite of calcium phosphate ceramics plus collagen membrane combined with delayed autologous bone marrow grafts was successful in regenerating large mandibular segmental defects.

In addition, Yuan *et al.*¹⁰ investigated in a **dog** unilateral, segmental defect model of the **mandibular body** osteogenically induced autologous bone marrow stromal cells (BMSCs) seeded onto natural corals (test) against natural corals without BMSCs (control). Healing was allowed for 12 or 32 weeks. For both healing periods, bony-union was achieved with BMSCs/coral constructs. Moreover, the shapes of repaired mandibles were very close to that of normal edentulous mandibles. On the contrary, natural coral without BMSCs was not associated with bony-union. Furthermore, in the test group bony-union was microscopically observed without clear boundary between newly formed bone and native bone. In addition, to evaluate the biomechanical properties of the repaired mandibles a three-point bending test was performed. After 12 weeks of healing, bending strength load in the BMSCs/coral group was 1.7 kN (SD: 0.4 kN), bending displacement

was 1.8 mm (SD: 0.4 mm), bending stress was 33.4 MPa (SD: 9.5 MPa) and Young's modulus was 507.6 MPa (SD: 194.9 MPa). For the contralateral normal edentulous mandible bending strength load was 2.4 kN (SD: 0.6 kN), bending displacement was 2.6 mm (SD: 0.5 mm), bending stress was 42.3 MPa (SD: 5.6 MPa) and Young's modulus was 239.6 MPa (SD: 71.8 MPa). Moreover, after 32 weeks of healing, bending strength load for the BMSCs/coral group was 1.5 kN (SD: 0.4 kN), bending displacement was 1.9 mm (SD: 0.8 mm), bending stress was 54.4 MPa (SD: 9.9 MPa) and Young's modulus was 433.0 MPa (SD: 159.5 MPa). Whereas for the contralateral normal edentulous mandible bending strength load was 1.8 kN (SD: 0.8 kN), bending displacement was 2.6 mm (SD: 0.6 mm), bending stress was 61.2 MPa (SD: 13.0 MPa) and Young's modulus was 292.9 MPa (SD: 113.6 MPa). Neither at 12 nor at 32 weeks bending strength load, bending displacement or bending stress were statistically significant different between tissue-engineered and normal mandibular bone. Since there was no bony union in the control (coral without BMSCs) group, three-point bending test could not be applied in this group. Quantitative data on bone ingrowths or scaffold degradation were not presented. The authors concluded that engineered bone from osteogenically induced BMSCs seeded onto biodegradable coral scaffolds can repair critical sized segmental mandibular defects in canines.

Moreover, Seto *et al.*¹⁸ investigated in a **monkey** unilateral, segmental defect model of the **mandibular body**, culture expanded autogenous bone marrow stromal cells (BMSCs) mixed with rhBMP-2 impregnated collagen sponges (test). After 24 weeks of healing, the combination graft of culture expanded BMSCs with rhBMP-2 soaked collagen sponges regenerated the mandibular bone completely. Additionally, histological evaluation revealed for rhBMP-2/BMSCs bone formation that reached the height of the host bone. Furthermore, the newly formed bone was remodeled and patterned lamellar bone, but it showed an irregular trabecular outline, its surface was rough and the regenerated bone seemed to be still immature. However, quantitative data on scaffold degradation or results of biomechanical testing were not presented. Finally, the authors suggested that culture expanded autogenous BMSCs mixed with rhBMP-2 impregnated collagen sponges is a promising new technique for bone regeneration in large bone defects.

Addendum No. 2

Busuttill Naudi *et al.*³⁰ investigated in a **rabbit** unilateral, segmental defect model of the **mandibular body** histological as well as biomechanical properties of rh-BMP-7 loaded onto β -TCP scaffolds (test) versus β -TCP scaffolds alone (control). In the test group, after three months of healing, next to complete bridging with woven as well as lamellar bone, union between the regenerated bone and the proximal bony segments was found. However, pattern of bone formation varied. In some cases a well-formed cortex was identified, whilst in others the new bone formation was primarily cancellous. In addition, in the test group the mean percentage regenerated bone volume was found to be 29% (SD: 6%), while the mean percentage for the control group was found to be 6% (SD: 3%). Moreover, in the mid surgical field, the bone volume of the test group was 23% (SD: 5%), whereas it was only 3% (SD: 2%) in the control group. Furthermore, it was reported that β -TCP resorbed more completely in the cases treated with rhBMP-7/ β -TCP. However, quantitative data on scaffold degradation were not presented. Biomechanical testing revealed for rh-BMP-7/ β -TCP failure moments of 0.06 to 2 Nm that were consistently greater than those treated with β -TCP alone (0 to 0.05 Nm). In addition, in some cases the mechanical properties of the regenerated bone were comparable to those of untreated bone. Finally, it was concluded that rh-BMP-7 loaded onto β -TCP scaffolds appeared to be an effective method for bone regeneration in the mandibular, critical size defect rabbit model.

Furthermore, in a **sheep** unilateral, segmental defect model of the **mandibular body**, Abu-Serriah *et al.*¹⁹ studied rh-BMP-7 conjoined with a bovine type-I collagen carrier. After 12 weeks of healing histological examination showed complete defect reconstruction. In principle, woven as well as lamellar bone that was entirely fused with the adjacent native bone was found. However, none of the newly formed bone showed restitution of the buccal and lingual cortices. Moreover, the newly formed bone was more porous and the Haversian systems were oriented at right angles to the direction of those of the native bone. Furthermore, histomorphometric measurements showed that the newly formed bone contained statistically significantly more marrow spaces (mean: 28.8%, SD: 11.5%) as compared to the native bone (mean: 3.5%, SD: 2.6%). Although all animals achieved bony union, a wide range of biomechanical properties was found. The rhBMP-7 induced bone reached a mean and median of 36% (range: 9 - 63%) of the strength of the bone of the non-operated, intact hemi-mandibles (NOS). The mean value of absorbed energy and stiffness of the rhBMP-7 induced bone were 61% and 24% of the NOS, respectively. These differences were statistically significant ($p < 0.05$). However,

while half of the samples of the rhBMP-7 induced bone had “weak” mechanical properties (9 – 25% strength of NOS) and a lower stiffness (6-18% of NOS); the other half showed relatively higher strength (47-63% of NOS) and was stiffer (35-47% of NOS). Unlike NOS, rhBMP-7 induced bone showed cracks that initiated at the superior border of the mandible and failed under tensile stresses. In addition, histomorphometric assessment revealed approximately 20% more porosity in less rigid as compared to rigid rhBMP-7 induced bone. Once again, quantitative data regarding scaffold degradation were not presented. Conclusively, the potential of rhBMP-7 as regards craniofacial bone reconstruction was supposed.

Similarly, Kontaxis *et al.*¹⁶ investigated rhBMP-7 loaded onto a bovine collagen type-1 carrier (test) in a **sheep** unilateral, segmental defect model of the **mandibular body**. Three months after surgery, complete bone regeneration occurred in all six test hemi-mandibles. However, quality as well as mechanical properties of the new bone were highly variable. In three out of six samples the newly formed bone contained fibrous tissue and was weaker and less stiff as compared to intact, non-operated hemi-mandibles (strength: 10-20%, stiffness: 6-15% of intact, non-operated hemi-mandibles). However, the other half of the test hemi-mandibles had “better-quality” bone and was significantly stiffer and stronger (strength: 45-63%, stiffness: 35-46% of intact, non-operated hemi-mandibles). These differences were statistically significant.

Moreover, in a **dog** unilateral, segmental defect model of the **mandibular body**, Toriumi *et al.*⁹⁷ studied inactive dog bone matrix carrier with rhBMP-2 (test) versus inactive dog bone matrix without rh-BMP-2 (control 1) or empty defects (control 2). Healing was allowed for 3 and 6 months. For both healing periods, inactive dog bone matrix carrier combined with rhBMP-2 was associated with bony-union. Furthermore, after 3 months of healing histomorphometric analysis revealed that 50% of the defect tissue was mineralized and that degradation resulted in only occasionally found islands of residual carrier material. Moreover, after 6 months of healing histomorphometric analysis revealed that 68% of the tissue occupying the defects was mineralized with only minimal evidence of residual carrier material. Additionally, the newly formed bone appeared normal, making it difficult to identify the point of transition from the inactive dog bone matrix carrier combined with rhBMP-2 to normal bone. On the other hand, inactive dog bone matrix without rh-BMP-2 was never associated with bony-union. After 3 as well as 6 months of healing, in principle fibrous scar tissue bridged the defect. In addition, in contrast to inactive dog bone matrix carrier combined with rh-BMP 2, relative large amounts of residual, nonviable bone matrix was found. Similarly, histological examination of

control group 2 (empty defects) revealed fibrous scar tissue with no bone formation at 3 as well as 6 months. Furthermore, biomechanical testing after 3 and 6 months revealed bending mean maximum moments in the test group of 7.4 Nm and 22.6 Nm, respectively. These values represented 9% and 27% of the mean maximum moment values of the contralateral (non-operated) hemi-mandibles for the 3 and 6 months specimens. In contrast, due to absence of bone, the mean maximum moments in the control 1 group (inactive dog bone matrix carrier without rh-BMP 2) were 0.0 Nm at both time points. Furthermore, biomechanical testing for control group 2 (empty defects) was not performed. Concisely, inactive dog bone matrix carrier with rhBMP-2 was associated with functionally stable mandibular reconstruction in the currently used dog model.

Furthermore, in a pilot study in an adult male **non-human primate (Macaca fascicularis monkey)** bilateral, segmental defect model of the **mandibular body**, Boyne²⁹ investigated rhBMP-2 loaded onto bovine tendon derived type I collagen carrier. After five months of healing bone bridging was found. Moreover, histological analysis revealed osseous repair with a trabecular outline, cortical bone structures as well as pattern of remodeling that may indicate bone stability and thus permanency of the regenerated area. However, beside these more qualitative data and presumptions, quantitative information of bone ingrowths, results of biomechanical testing or data regarding scaffold degradation were not given. Nonetheless, it was concluded that rhBMP-2 - without the use of bone grafts - might be a powerful inductor of osseous regeneration in large discontinuity mandibular defects in non-human primates.

Likewise, Boyne¹⁰¹ studied in a middle-aged as well as in an aged, adult male **non-human primate (Macaca fascicularis monkey)** unilateral, segmental defect model of the **mandibular body**, rhBMP-2 loaded onto a collagen carrier. After four and six months of healing histologic examination revealed, bone bridging, excellent bone regeneration as well as good bone remodeling and thick outer cortex formation in the middle-aged group. Moreover, six months after implantation of rhBMP-2, there was no gross or microscopically discernible difference in the amount or quality of bone formation in the younger or older animals. In fact, in numerous cases, more bone had formed in the older animals. Obviously, aging appeared to have no effect on the osteoinductive effect of rhBMP-2. Again, beside these more qualitative data, quantitative information of bone ingrowths, results of biomechanical testing or data regarding scaffold degradation were not given. Finally, it was suggested that the use of rhBMP-2 - without bone grafting materials - will offer a new method of osseous reconstruction in clinical facial bone defects.

For instance, Zhou *et al.*¹⁰⁹ compared in a **non-human primate (Rhesus monkey)** bilateral, segmental defect model of the **mandibular body**, mandibular reconstruction with prefabricated, vascularized tissue-engineered bone flaps with rhBMP-2 (i.e.: demineralized freeze-dried bone allograft carrier loaded with rhBMP-2 (P-DFDBA-BMP) or coralline hydroxyapatite loaded with rhBMP-2 (P-CHA-BMP) implanted in the latissimus dorsi muscle for prefabrication) and rhBMP-2 in situ (i.e.: DFDBA loaded with rhBMP-2 (S-DFDBA-BMP) or CHA loaded with rhBMP-2 (S-CHA-BMP) directly applied to the segmental defect). At 26 weeks after implantation the mandibular defects were reconstructed successfully with P-DFDBA-BMP, P-CHA-BMP as well as S-CHA-BMP. However, this was not the case for S-DFDBA-BMP. With S-DFDBA-BMP implants, half of the area of the defect was occupied by bone regenerated from the two ends to the center, but the center of the defect was occupied by soft tissues and thus the discontinuity of the mandible remained. However, mechanical testing of the restored jaw segments was not done. Moreover, for the P-CHA-BMP and S-CHA-BMP groups the CHA blocks were partly absorbed, whereas for P-DFDBA-BMP as well as S-DFDBA-BMP the blocks of DFDBA had disappeared completely. Apparently, in the current primate model DFDBA, which is mainly composed of insoluble, highly cross-linked type I collagen, might not be a favorable carrier for the in-situ application of rhBMP-2.

Furthermore, Seto *et al.*¹⁰² evaluated in a **non-human primate (Japanese monkey)** unilateral, segmental defect model of the **mandibular body**, reconstruction with an rhBMP-2/polyglycolic co-lactic acid (PGLA) complex and autogenous bone marrow in different ratios. Therefore, rhBMP-2 was suspended in a solution of PGLA and lyophilized to make an rhBMP-2/PGLA complex. Thereafter, the rhBMP-2/PGLA complex combined with autogenous bone marrow in ratios of 3:0, 2.5:0.5, 2:1 or 0:3 (i.e.: vol:vol) were implanted into mandibular continuity defects. Sixteen weeks after surgery, the implantation of rhBMP-2/PGLA alone resulted in the formation of only small amounts of bone in 2 out of 3 monkeys and was associated with the infiltration of fibrous tissue into the bone defects. Similarly, the 2.5:0.5 combination of rhBMP-2/PGLA and bone marrow, although it was associated with larger amounts of new bone, did not generate complete bony union in 2 of 3 monkeys. Conversely, in all monkeys implanted with the 2:1 or the 0:3 mixtures of rhBMP-2/PGLA and bone marrow complete bone formation in the site of the mandibular continuity defects as well as complete bone bridging was established. However, results of biomechanical testing or data regarding scaffold degradation were not given. Moreover, the biologic activity of rhBMP-2 within the

rhBMP-2/PGLA polymer was not tested. Thus, the substantiation for judgment of the authors that it is not clear whether BMPs will definitely induce bone formation in humans is somewhat speculative.

In a **sheep** unilateral, segmental defect model of the **mandibular body**, Ayoub *et al.*¹⁷ applied rhBMP-7 in a bovine collagen type I carrier wrapped into a sterno-occipitalis muscle flap. Three months after surgery, bridging of the surgical defect was found in only 3 out of 5 animals.

Addendum No. 3

Kokemueller *et al.*¹¹⁹ restored an unilateral, segmental defect of the mandibular body in a patient with prefabricated, vascularized bioartificial bone grafts. The patient was a 57-year old man, who had suffered from chronic osteomyelitis for many years and finally underwent continuity resection with loss of his left hemimandible. For prefabrication of vascularized bioartificial bone grafts, three bone biopsies from the iliac crest were harvested, morselized and mixed with amorphous bone marrow aspirate from the depth of the biopsy areas. After dissection of the latissimus dorsi muscle on the patient's left side and preparation of perforating vessels from the thoraco-dorsal trunk, four blood soaked β -TCP cylinders were supplied with a central vascular bundle and loaded with the harvested osteogenic material. Six months thereafter, the prefabricated, vascularized bioartificial bone grafts were transplanted into the mandibular continuity defect area and kept in place by a titanium mesh. There were no complications regarding postoperative recovery. Twelve months after surgery, there were no signs of infection or rejection at the site of transplantation. However, bone histology or data regarding scaffold degradation were not presented. Finally the authors assumed that prefabricated, vascularized bioartificial bone grafts have the potential to change the current principles of bone transplantation and that they may serve as a new therapeutic option in the future.

Comparable results were reported by Warnke *et al.*^{113, 114} after mandibular reconstruction of one patient with a prefabricated, vascularized bioartificial bone graft. For prefabrication, xenogeneic bone mineral blocks as osteoconductive carrier, autologous bone marrow from the iliac crest and rhBMP-7 loaded onto bovine collagen type I were combined.

Furthermore, Herford and Cicciù¹²⁰ reconstructed, after en bloc resection of a giant cell tumor, a segmental defect of the mandibular body in a 25-year-old patient by using autogenous hip bone transplants screwed onto a titanium reconstruction plate covered by a rhBMP-2 containing collagen sponge. After three months of healing, clinical palpation of the mucosa overlying the resected area displayed the

hard indurated calcifying surface of the regenerated bone. Moreover, six months postoperatively radiographic evidence of mandibular continuity was found. However, bone histology was not presented. Somehow speculative, as Herford and Cicciù¹²⁰ combined rhBMP-2 with autogenous hip bone, bone growth cytokines were considered as predictable alternative to traditional grafting techniques.

Likewise, Herford *et al.*¹¹⁵ reported in an 18-year-old patient comparable effects for the combined therapeutic use of autogenous iliac crest bone and rhBMP-2. Additionally, Carter *et al.*¹¹⁷ published for autogenous bone marrow combined with allogenic bone as osteoconductive scaffolds and rhBMP-2 impregnated collagen sponges similar results in two patients. However the published outcomes were not uniform. It should be highlighted that in one case of Carter *et al.*¹¹⁷ autogenous bone marrow combined with allogenic bone and rhBMP-2 impregnated collagen sponges was not associated with bony union.

After en bloc resection of a dentinogenic ghost cell tumor, Cicciù *et al.*¹²¹ restored an unilateral, segmental defect of the mandibular body in an 18-year-old patient by rhBMP-2 loaded onto collagen mixed with mineralized or demineralized allograft bone as osteoconductive scaffold. After three months of healing, clinical palpation of the mucosa overlying the resected area displayed the hard indurated calcifying surface of the regenerated bone. Moreover, radiographic evidence of mandibular continuity was presented after nine months of healing. Accordingly, nine months postoperatively the titanium mesh was removed and dental implants were placed. However, bone histology or data regarding scaffold degradation were not presented. Finally the authors speculated that for osseous defect regeneration, the use of exogenous cytokines, particularly those in the BMPs series, will become common.

Comparable outcomes for rhBMP-2 loaded onto collagen sponges without the additional application of osteoconductive bone allografts were reported by Herford *et al.*¹¹⁵ as well as Herford and Boyne¹¹⁸. However, the outcomes were not consistent. It should be underlined that in one case of Carter *et al.*¹¹⁷ rhBMP-2 loaded onto absorbable collagen sponges did not induce bone formation.

Moreover, Clokie and Sandor¹¹⁶ reconstructed segmental defects of the anterior mandible (one case), the mandibular body (five cases), the ramus of the mandible (one case) as well as the combination of ramus and body of the mandible (three cases) in a total of ten patients with rhBMP-7 mixed with osteoconductive demineralized bone matrix in a reverse-phase medium (BMP bioimplant). However, the paper of Clokie and Sandor¹¹⁶ is somehow inconsistent. For example, they report that all patients were followed for a minimum of nine months and that all patients

demonstrated clinical as well as radiographic evidence of restored mandibular continuity, but they also state that radiographic evidence of bone formation was not fully evident until one year after reconstruction. Additionally, according to the authors, both functional and esthetic results were comparable if not superior to those achieved with autogenous bone grafting, but bone histology or data regarding scaffold degradation were not presented.

Similar effects for native human BMPs delivered in an allogenic demineralized bone matrix gel (Moghadam *et al.*¹¹¹) as well as xenogeneic BMPs combined with an allogenic bone matrix as delivery system (Ferretti C and Ripamonti U¹¹²) have been published. However, it should be mentioned that only two out of six patients of Ferretti C and Ripamonti U¹¹² treated with xenogeneic BMPs combined with an allogenic bone matrix as carrier demonstrated histological evidence of osteogenic activity or radiographic signs of ossification.

SUBCUTANEOUS TISSUE RESPONSE TO TITANIUM, POLY(ε-CAPROLACTONE) AND CARBONATE-SUBSTITUTED HYDROXYAPATITE-COATED POLY(ε-CAPROLACTONE) PLATES: A RABBIT STUDY

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Introduction

In previous studies, a titanium modular mandibular endoprosthesis has been developed to repair segmental defects of the mandible, which demonstrated stability and good fixation to bone in animal studies.^{1,2} However, in some instances, dehiscence has proven to be a problem especially when the soft tissue does not adhere to the body of device, with subsequent exposure of the prosthesis.³

It is known that implant surface chemistry, shape, and mechanical properties are all factors that affect the implant-soft tissue adhesion.³ For example, implant surface topography has a major effect on implant tissue response.⁴ Smooth surfaced implants result in a foreign body reaction, which is characterized by fibrous tissue encapsulation of the implant and the presence of inflammatory cells at the implant-soft tissue interface. On the other hand, implant surface roughness can have a favorable effect on the soft tissue response. It has been suggested that implants with a surface roughness value of 3.3 microns or larger tend to become infiltrated with inflammatory tissue, while implants with a surface roughness value 1 to 2 microns porosity appear to allow direct fibroblast attachment to the surface, which is supposed to be independent of the physico-chemical nature of the implant surface.⁴ Although the relationship between material surface topography and cellular behavior is complex and still not fully understood, Unadkat *et al.*, hypothesized that changes in the surface topography can affect cellular responses to a material by mimicking the influence and action of growth factors.⁵ Substrate surface features have been shown to induce significant modulation of focal adhesion formation, cytoskeletal development, and cellular spreading, changes that are subsequently transduced to signaling pathways, affecting functional differentiation through integrin-specific signaling pathways.⁶ Surface roughness and total surface area of an implant can be favorable for increased cell adhesion and migration as well as the production of extra cellular matrix (ECM). In view of this, implant surfaces with a texture such as nodes, pores, grooves or random patterns are often associated with a marked change of cell morphology, cell activity and cellular production of autocrine as well as paracrine regulatory factors compared to smooth surface.⁷

Implant surface roughness can be created by adding or subtracting material from the implant surface. Addition of material can be done by a coating procedure^{8,9} i.e. titanium plasma spraying and subtraction can be done by grit-blasting¹⁰⁻¹² or etching procedures^{13,14}. Most of the studies to date have investigated the effect of increased surface roughness of the implant on bone regeneration. However, there were less studies investigating the effect on soft tissue attachment. Lee *et al.*, 2010, designed

an experiment to study the effect of titanium surface modification on soft tissue attachment.³ Both machine surfaced and etched titanium bullets were implanted for 6 months in the muscle of *Macaca fascicularis* monkeys. The histological results showed a lack of direct contact between muscle tissue and machined titanium implant surface. Also, surface etching did not result in a significant improvement to the soft tissue attachment compared to the machined titanium surface.³

Although titanium is preferred for bone reconstruction due to its mechanical strength and ability to withstand long-term loading, tissue adaptation to titanium surface is still limited. The soft tissue response to an implant material is also dependent on the mechanical properties of the biomaterial.¹⁵ In general, less stiff biomaterials improve the soft tissue response. The mechanical properties of polymers are easier to fine-tune in order to get a better soft tissue response than metals, like titanium. A candidate material, as can be used for the fabricating of a modular endoprosthesis with an improved soft tissue adaptation, is poly(ϵ -caprolactone) (PCL). This material has several advantages over other polymers. It is more stable in ambient conditions, significantly less expensive and is readily available in large quantities.^{16,17} In addition, PCL can be easily combined with other materials in order to further formulate the tissue response. Active screen plasma surface modification has been shown to improve osteoblast cell adhesion and spreading on PCL surface¹⁸, while chemical hydrolysis to introduce carboxylate groups onto the surface of the PCL was found to improve surface wettability and roughness of the PCL, which was correlated with increased cell attachment.¹⁹ Several techniques are available to manufacture an implant from PCL. One of the approaches is laser sintering, where small PCL particles are selectively fused layer-by-layer by a high power laser to build a three-dimensional (3D) device. This method allows adaptation of the mechanical properties of the final implant.²⁰ Selective laser-sintered (SLS) and solid free-form fabrication (SFF) manufactured PCL scaffolds with a porosity between 37–55% were reported to have mechanical properties comparable with human trabecular bone. The compressive modulus of such scaffolds was found to be within the 52–68 MPa range, and the ultimate compressive strength was within the 2.0–3.2 MPa range, which makes this material an attractive substitute for human bone and its application in for bone reconstruction in load bearing areas.²⁰

For the current study, we hypothesized that implants made of PCL would lead to better soft tissue adaptability and adhesion than commercially pure titanium implants. In addition, we supposed that surface roughening, created by the deposition of a CHA coating on PCL, would further improve the soft tissue response. Therefore, implants were incubated in modified Simulated Body Fluid

(mSBF), which resulted in the nucleation and growth of a CHA coating on the implant surface.

A subcutaneous rabbit model was used to study the soft tissue response. Analysis after 5 weeks of implantation was based on a tissue peel test to determine the force required to separate the soft tissue from the various implant surfaces and on light microscopy examination.

Materials and Methods

Implant Materials

Non-coated PCL, CHA coated PCL, and commercially titanium (Ti) implants were manufactured. The implants were rectangular-shaped, measured 10 x 5 x 3 mm, and were provided with rounded off corners and edges. The PCL implants were fabricated via laser sintering as previously reported.²⁰ The PCL implants were used as-received or were coated with CHA by incubation at 37°C in mSBF for 8 days under continuous rotation. Prior to mSBF incubation, the PCL plates were hydrolyzed in a 1 M NaOH for 60 min. After hydrolysis, plates were rinsed and incubated in the mSBF. The mSBF solution has a similar composition to that of human plasma and also to that of the SBF solution reported by Kokubo *et al.*, but with double the concentration of calcium and phosphate to enhance mineral growth, and was prepared as previously reported.^{21, 22} Briefly, 141mM NaCl, 4.0mM KCl, 0.5mM MgSO₄, 1.0mM MgCl₂, 4.2mM NaHCO₃, 5.0mM CaCl₂, and 2.0mM KH₂PO₄ in deionized ultra-filtered water, pH was adjusted to 6.8 with 2N HCl or 2N NaOH.

Prior to their use in the *in vivo* study, PCL and CHA coated PCL implants were sterilized by ethylene oxide and Ti plates were sterilized by autoclave.

Animal Model and Implantation Procedure

The animal experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Singhealth, Singapore. The animal laboratory was certified by the International Association for Assessment of Laboratory Animal Care (IAALAC).

Nine female New Zealand white rabbits, 3–4 months old, were used in this study. The surgery was performed under general anesthesia by intramuscular injection of 1.5 mg Ketamine (Parnell Laboratories, Alexandria, Australia). Anesthesia was maintained by 1-1.5% isoflurane gas through a mask with constant volume ventilator. Heart rate and oxygen saturation were monitored during the procedure.

Prior to surgery the skin was shaved, washed and disinfected with povidone iodine 1% solution, Hexodane 0.5% (Chlorhexidine 0.05% W/V in Methylated Spirit 70%) and centrimide 1% solution. Each animal was given a unique code. Six longitudinal incisions of about 1.5 cm were made at the left and right side of the vertebral column at 3 cm apart of each other. Six subcutaneous pockets were prepared by blunt dissection with scissors. Each of the pockets in each animal received one of the 3 types of implants. A randomization schedule was made for implant allocation, which listed the animal's code and the corresponding subcutaneous pocket number (1–6) in each animal.

Six implants (2 PCL, 2 CHA coated PCL and 2 Ti) were inserted into each rabbit. With 9 rabbits used, a total of fifty four implants were inserted (18 PCL, 18 CHA coated PCL and 18 Ti). After implant installation, the wounds were closed using 3-0 Vicryl® intracutaneous sutures (Ethicon Inc., Somerville, NJ, USA). After 5 weeks, all animals were euthanized and the implants with surrounding tissues (a rectangular patch of the skin encompassing each plate) were harvested.

After harvesting, the retrieved specimens were divided into two equally-sized groups, i.e., one group was used for testing of the soft tissue adhesion strength and the other was used for histological analysis.

Peel-Test Procedure

Immediately after harvesting of the plates, 27 samples (9 of each plate type) were subjected to a soft tissue peel-test using an Instron® 8800 microforce tester (Instron Corporation, Satec™, Norwood, MA, USA) equipped with a static load cell with a capacity of 10 N.

All specimens were prepared before installation into the Instron® machine (Fig. 1), i.e., one-third of the plate surface was exposed to allow its grip by the lower grip of the machine, while the remaining two-third of the attached tissue was kept intact to the plate for peel test. One end of the soft tissue was attached vertically to the upper grip of the testing machine and the plate surface was kept parallel (180 degree) to the tension force. A tension force was applied with the top upper arm of the machine, which was moving upwards at a speed of 5 mm/min. The test was carried out until tissue was completely peeled off from the plate surface. Mechanical data were recorded and the corresponding force-displacement curves were generated. The values of maximum force attained were then averaged and the standard deviations were calculated. After performance of the peel test, the specimens were fixed in 10% formaldehyde for further evaluation with scanning electron microscopy (SEM).

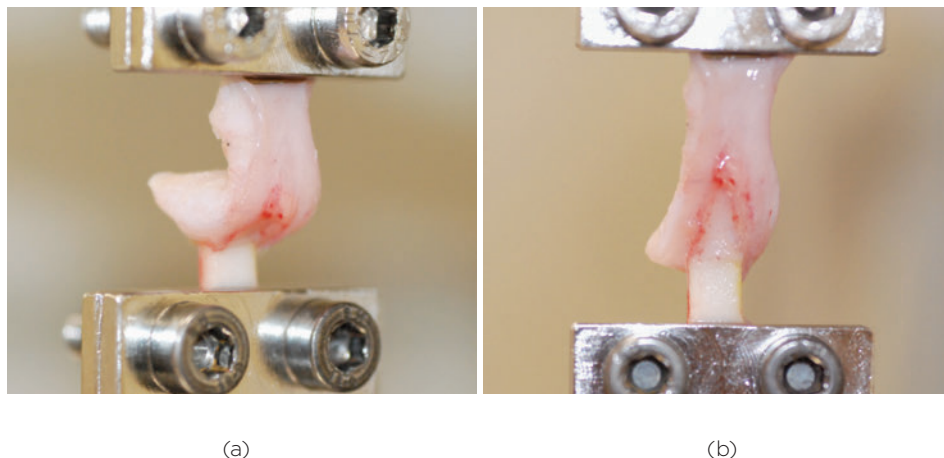


Figure 1. Specimens were installed in Instron® 8800 machine for tissue peel test; (a) before and (b) during peel test for PCL plate

Scanning Electron Microscopy (SEM)

The morphology of the plates before and after implantation was investigated by SEM. PCL and CHA coated PCL plates were mounted on aluminum stubs and sputter coated with a thin layer of gold. Samples were imaged under high vacuum using a Philips XL30 FEG scanning electron microscope (Hillsboro, Oregon, USA) operating at 10 kV. Ti plates were mounted on stub and examined at 20 kV without gold sputter coated.

Histological Analysis

Before histological preparation, the specimens with their surrounding tissues were immersed for 1 week in buffered 10% formalin solution for fixation (ICM Pharma Pte Ltd, Singapore), then dehydrated in a graded series of alcohol and embedded in methylmetacrylate (MMA). After polymerization, the tissue blocks were mounted in a modified inner circular saw microtome (Leica® RM 2165, Wetzlar Germany). At least, three histological sections were made from each specimen. Sections had a thickness of 10-15 μm and were stained with methylene blue/basic fuchsin and examined using a light microscope (Olympus® U-D03, Tokyo, Japan)

All sections were observed and independently scored by two blinded observers (N Chanchareonsook and Lee S) using an established soft tissue histology grading scale²³, as shown in Table I. When the two observers disagreed on a score, the section was discussed until a consensus was reached. The thickness of capsule around implants was measured in micrometers (μm) using 'Cell' digital imaging software

program (Olympus®, Germany). Subsequently, the means of capsule thickness of each implant type were calculated.

Table I. Soft Tissue Histologic Grading Scale
(adapted and modified by Jansen *et al.*, 1994)

Evaluation	Response	Score
Capsule qualitatively	Capsule is fibrous, mature, not dense, resembling connective or fat tissue in the non-injured regions	4
	Capsule tissue is fibrous but immature, showing fibroblasts and little collagen	3
	Capsule tissue granulous and dense, containing both fibroblasts and many inflammatory cells	2
	Capsule consists of masses of inflammatory cells with little or no signs of connective tissue organization	1
	Cannot be evaluated because of infection or other factors not necessarily related to the material	0
Interface qualitatively	Fibroblasts contact the implant surface without the presence of macrophages or leucocytes	4
	Scattered foci of macrophages and leucocytes are present	3
	One layer of macrophages and leucocytes are present	2
	Multiple layers of macrophages and leucocytes are present	1
	Cannot be evaluated because of infection or other factors not necessarily related to the material	0

Statistical Analysis

Data from the peel-test and histological measurements were statistically analyzed using SAS 9.2 statistical software (SAS Institute Inc, Cary, North Carolina). Measurements were evaluated by Analysis of Variance (ANOVA) with pair-wise comparison post test to identify the groups that differed from each other. This was done with no correction for the Type I error rate across the pair-wise tests. A *P*-value < 0.05 was considered significant.

Results

Clinical Examination

At 5 weeks after surgery, all rabbits tolerated the implant installation very well. Tissue necrosis was observed in only one animal in the area where Ketamine was injected. This site was close to a CHA coated PCL plate. Therefore, this specimen was subsequently excluded from further analysis to avoid an effect on the experimental results. In all other animals, the surgical sites presented good healing without any wound dehiscence. All plates were palpable through the skin. It appeared that some of the plates had migrated from the insertion site. The Ti-plates had migrated over a distance of 0-4 cm, while CHA coated and non-coated PCL plates had migrated by 0-1 cm.

Peel-Testing

Two of the 27 samples were excluded from the peel-test study. One CHA coated PCL was excluded due to necrosis of skin from the effect of Ketamine injection and one Ti plate sample was excluded due to formation of hematoma on the plate surface during tissue manipulation at tissue harvesting.

The average energy used for the peel test for Ti, PCL and CHA coated PCL was 0.728×10^{-3} , 0.543×10^{-3} and 0.274×10^{-3} J respectively. The average peel force for Ti, PCL and CHA coated PCL was 0.17, 0.104 and 0.098 N respectively. (Fig. 2)

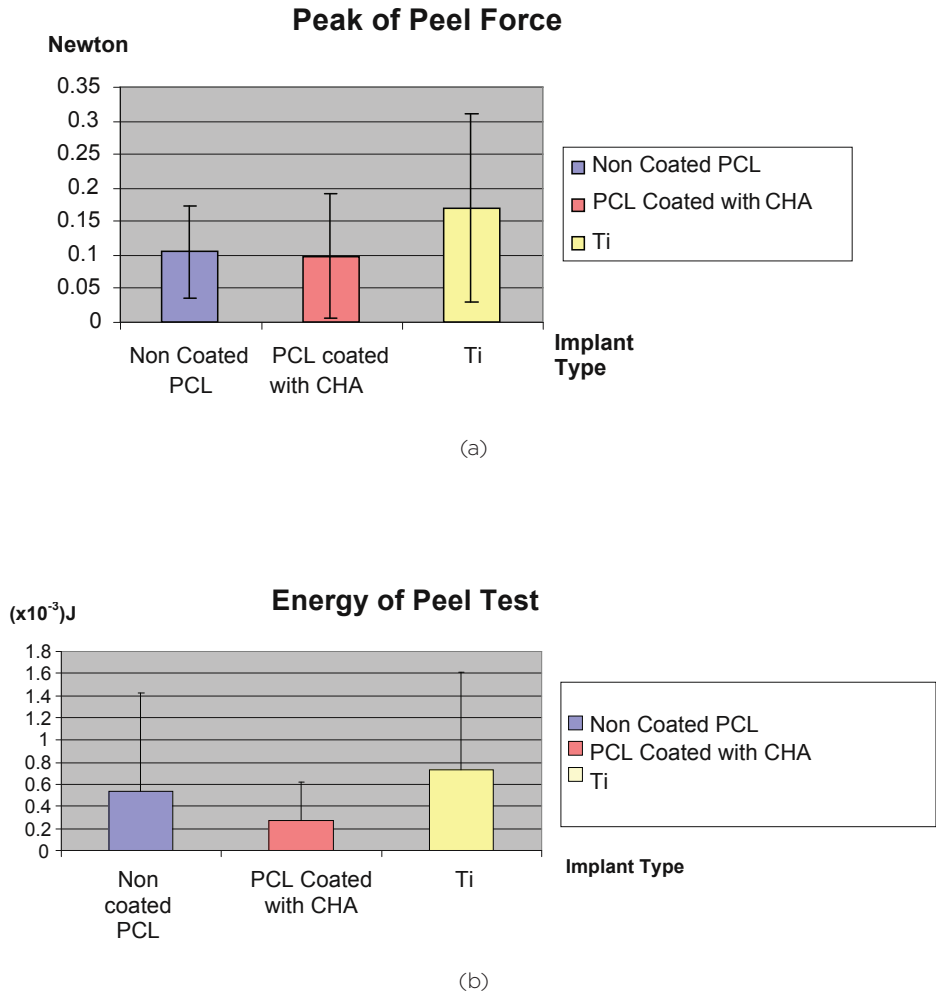
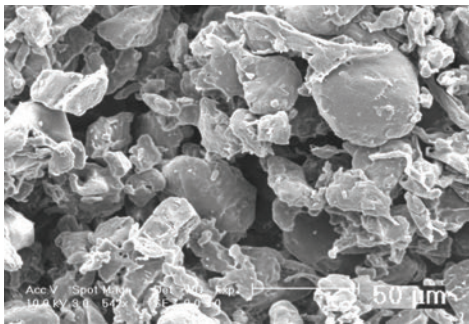


Figure 2. Peel test analysis of machined surface Ti implant, non-coated PCL implant and PCL surface coated with carbonate-substituted hydroxyapatite (CHA).

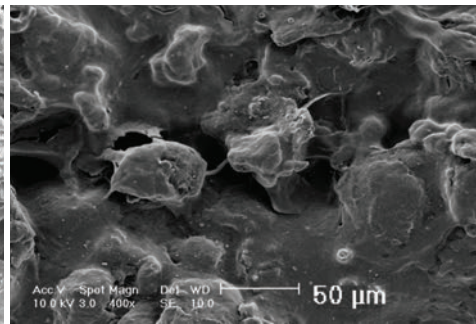
Surface Morphology

It is shown from SEM images that the non-coated PCL implants have rougher surface appearance than those of the Ti implants. The CHA coating, as deposited on the PCL implants, had a microscale plate-like morphology and increased the surface roughness of the PCL implants compared with the non-coated ones. After 5 weeks of implantation, the surfaces of the Implants after the peel test were not altered considerably compared to images before implantation (Fig.3). There were no remaining tissue and cells visible on all plate types after the peel test.

Non-coated PCL

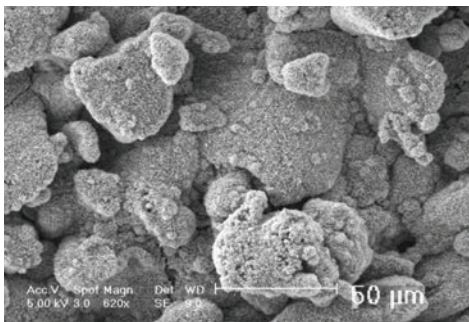


(a) Before

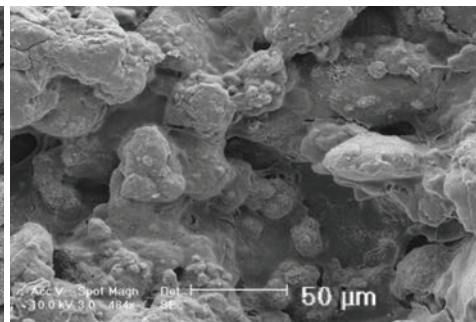


(b) After

Coated PCL



(c) Before



(d) After

Ti

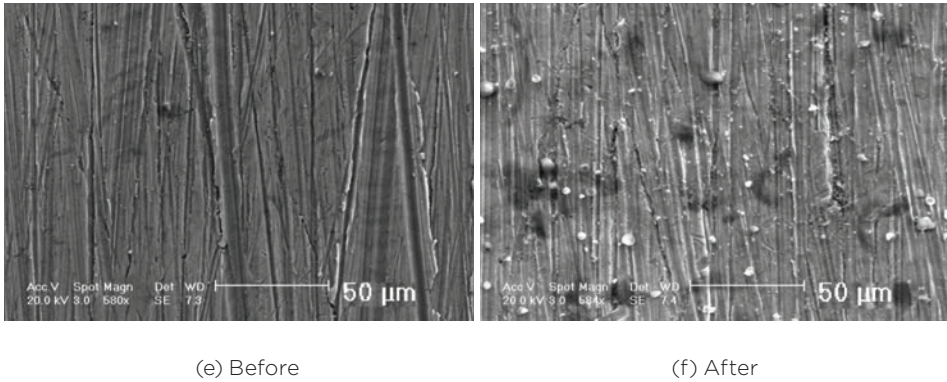


Figure 3. Scanning electron microscopy (SEM) images of Ti-plates, coated and non coated PCL plates before and after 5 weeks of subcutaneous tissue implantation in the rabbit model: a) non-coated PCL before surgery, b) non-coated PCL after implantation and peel test, c) coated PCL before surgery, d) coated PCL after implantation and peel test, e) machined-surface titanium plate before surgery and f) machined-surface titanium plate after implantation and peel test. The images demonstrated the rough surface of each implant type, i.e. the excellent pattern of microscale plate-like morphology of coated PCL as well as the machined surface appearance of the Ti-plates. The implant surfaces in all type did not show any remnant of connective tissue as left on the surface.

Histological Analysis

Evaluation of the histological sections revealed a fairly uniform tissue response for the 3 types of implant. In all sections, normal skin and underlying tissues, including fat tissue, could be observed. The surface of both CHA coated and non-coated PCL plates appeared to be rougher compared with those of the Ti implants. The CHA layer on the coated PCL implants could easily be identified and was visible a thin red layer on the outer surface of these implants.

All implants were found to be surrounded by a fibrous tissue capsule. This capsule was about 7 to 8 cell layers in thickness for PCL and CHA PCL coated implants and 14 to 17 cell layers for Ti- implants. The capsule had an aligned morphology with collagen bundles running parallel to the implant surface. Occasionally inflammatory cells were seen in the interface between capsule and implant surface. The presence of inflammatory cells was more evident for the PCL implants compared with the titanium implants (Fig.4).

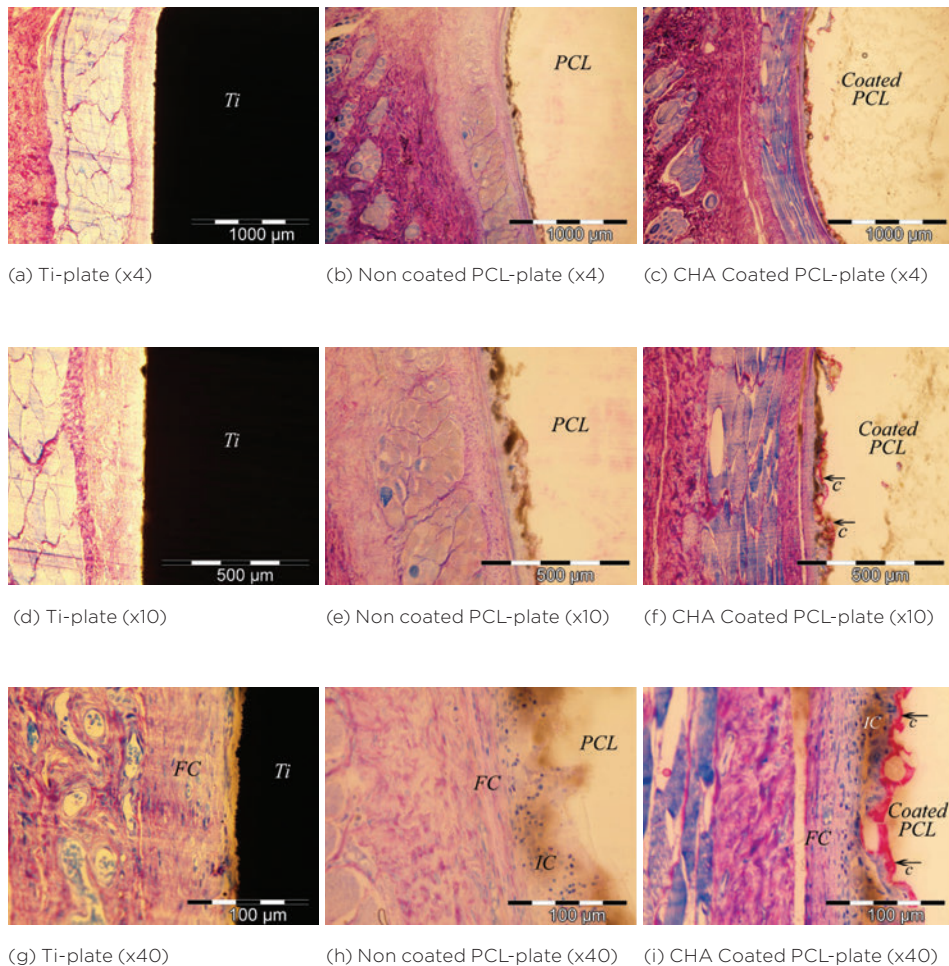


Figure 4. Histological images of non-coated PCL plates, coated PCL plates and Ti-plates after 5 weeks of subcutaneous tissue implantation in the rabbit model *Ti*: Titanium implant; *FC*: Fibrous capsule; *PCL*: Polycaprolactone implant; *C*: carbonate-substituted hydroxyapatite (CHA) Coating; *IC*: Inflammatory cells (Methylene blue and basic fuchsin) a) to c) magnification of x4, d) to f) magnification of x10 and g) to i) magnification of x40.

Histomorphometry

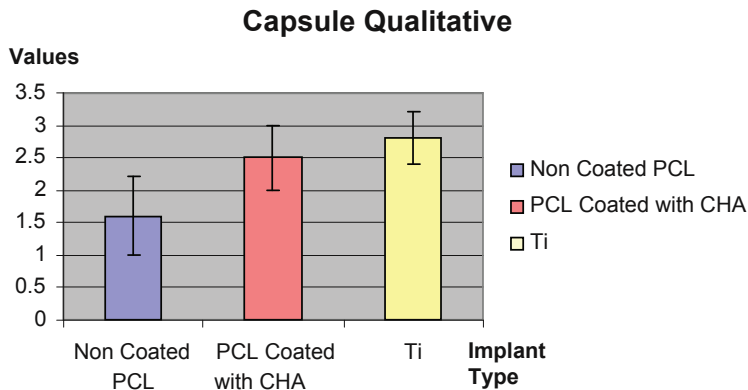
The average capsule thickness around the PCL implant was $34.3 \pm 15.5 \mu\text{m}$, around CHA coated PCL $50.8 \pm 16.4 \mu\text{m}$ and around the Ti-plate was $62.2 \pm 15.7 \mu\text{m}$. Statistical testing revealed that the capsule around the non-coated PCL plate was significantly thinner compared with the CHA coated PCL and Ti plates (Table II). Also, the capsule around the CHA coated PCL plates was found to be significantly thinner compared to that on the Ti plates (Table II).

Table II. The average capsule thickness and Pair-wise comparisons between implant type. The result showed significant different with respect to capsule thickness.

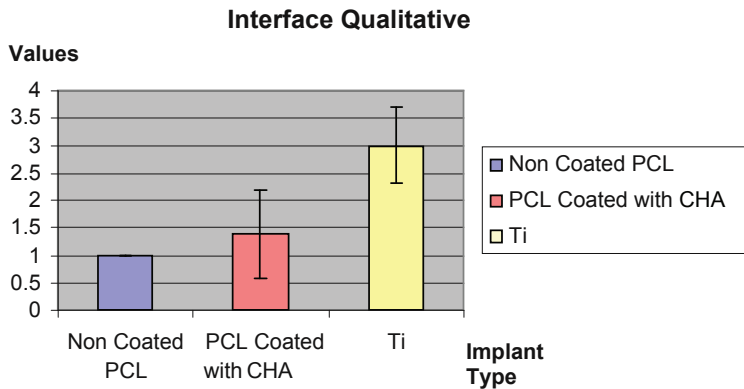
Type of plates	Mean of capsule thickness (Qm) (\pm SD.)	Pair-wise Comparisons between Means		
		Non-coated PCL	CHA Coated PCL	Ti
Non coated PCL	34.3(\pm 15.5)	-	0.0003**	<.0001**
CHA Coated PCL-plate	50.8(\pm 16.4)	0.0003**	-	0.0101**
Ti	62.2(\pm 15.7)	<.0001**	0.0101**	-

**Statistically significant difference ($p \leq 0.05$)

The soft tissue grading scores for capsule quality and interfacial tissue response are depicted in Figure 5. The mean soft tissue grading score for capsule quality of PCL, CHA coated PCL and Ti implants are 1.6 ± 0.6 , 2.5 ± 0.5 and 2.8 ± 0.4 respectively. The mean soft tissue grading score for interface quality of PCL, CHA coated PCL and Ti implants are 1.0 ± 0.0 , 1.4 ± 0.8 and 3 ± 0.7 (Fig. 5).



(a)



(b)

Figure 5. Grading scale scores of capsule quality and interface quality for PCL, CHA-coated PCL and Ti implants. The comparison of percent score distribution of capsule quantity, capsule quality and interface quality between implant types were analyzed by pair-wise comparison. The Significant differences for capsule quality were found between non-coated PCL vs PCL coated with CHA ($p < .001$), non-coated PCL vs Ti ($p < .001$) and PCL coated with CHA vs Ti, ($p = .042$). Significant differences for capsule interface quality were found between non-coated PCL vs PCL coated with CHA ($p = .010$), non-coated PCL vs Ti ($p < .001$) and PCL coated with CHA vs Ti ($p < .001$).

Statistical analysis by Fisher's exact test on global null hypothesis testing showed homogeneity of data distribution for all three implant groups. The comparison of percent score distribution of capsule quality and interface quality between implant types was analyzed by Pair-wise comparisons. The results show that all three groups differed significantly relative to capsule quality and capsule interface quality.

Discussion

Soft tissue adherence between host tissues and an implant is important to minimize implant-soft tissue dehiscence, to improve the long-term performance of a device in vivo and to reduce the occurrence of infection. Metallic and polymeric implants are known to become surrounded by a fibrous tissue capsule after their installation in soft body tissue.^{3, 24, 25} Physical properties such as implant shape, mechanical properties of the implant material, and degree of surface roughness as well as chemical properties determine the final soft tissue response.³

In this study, rectangular plates composed of different materials, i.e.,

commercially pure titanium (Ti), non-coated PCL and CHA coated PCL were inserted subcutaneously into the back of rabbits for 5 weeks. It was hypothesized that: (1) the PCL implant would show an improved soft tissue response compared to the Ti implant, and (2) the CHA coating, as provided to the PCL plates, would further favor the soft tissue reaction. However, histological analysis after retrieval of the implants did not confirm the hypothesis. Overall, the soft tissue response to all implants was very similar and no direct attachment of connective tissue to the various surfaces was observed.

Clinical observation of the implants after 5 weeks of installation in the rabbits showed that the machined surfaces Ti plates had migrated over a distance of 0–4 cm. This was more than the coated and non-coated PCL plates, which were found to have migrated 0 to 1 cm. Such migrational behavior is commonly found when implants are inserted in soft tissue without any additional fixation to the soft tissue layer and is related to the soft tissue adhesion of each particular implant surface. The degree of migration indicates a lack of soft tissue adhesion of the implant surface. The current findings corroborate with an earlier study dealing with the migration of microchips in Beagle dogs.²⁶ In this study, microchips made of 3 different materials, i.e., bioglass, acid-etched bioglass and bioglass provided with a polypropylene cap were installed in the soft tissue around head and shoulder of the Beagle dogs for 16 weeks. Different degrees of microchip migration were observed, which was depending on the location and the used material. The microchips in the shoulder, which had more muscle activity, showed a migration up to the maximum of 11 cm. In contrast, the microchips in the head area moved only to a maximum distance of 2 cm from their insertion point. Further, the microchips made of etched bioglass or provided with a polypropylene cap were found to migrate significantly less than microchips made of just bioglass.²⁶

To define the level of soft tissue attachment with the implant surface, we made use of a tissue peel test. Bobyn JD *et al.*²⁷, found that the increased strength of tissue attachment is correlated with implantation time. Similarly, in an earlier pilot study, we demonstrated that specimens at 2 weeks of plate implantation (result not shown) demonstrated a poor soft tissue attachment irrespective of the implant surface finish. Therefore in the current study, the implantation time was increased to 5 weeks to allow for the maturation of the tissue attachment. Subsequently, the peel test data demonstrated that there was no significant difference in peel test readings between the different implant surfaces and therefore no relevant mechanical effect of implant surface preparation on soft-tissue bonding was observed. However, we found that during the peel test, tissue adhesion between implant and fresh subcutaneous rabbit

specimen was fragile and the peel test required highly delicate tissue manipulation especially when the samples were small in size. As a consequence, the protocol for the tissue peel test still has room for improvement in future studies. Furthermore, the pores and channels in the PCL structure allowed a better maintenance of moisture than the titanium implant. This can inadvertently affect the peel test result, as faster desiccation of the soft tissue on the titanium implant during testing may lead to increased adhesion. In future experiments, environmental control of moisture and temperature should be regulated to ensure that the soft tissue specimens remain in their optimal condition.

No previous studies are available, in which a peel test was done for PCL. Overall, our peel force results were found to be lower compared with previous studies.^{27, 28, 29} Hacking *et al.*²⁸, studied fibrous tissue ingrowth and attachment to porous tantalum after insertion in the dorsal subcutaneous tissue in dogs. A peel test was done using a servo-hydraulic tensile test machine at a rate of 5 mm/min. Peel force at 4, 8, and 16 weeks was reported at 61, 71, and 89 g/mm respectively.²⁸ Zhao D *et al.*²⁹, studied titanium fiber mesh with 84.7% porosity and compared this material with conical implants coated with various compositions of bioactive glass. Ti mesh was inserted into the dorsal subcutaneous soft tissue and muscles in the back of rats for 8 weeks. Titanium fiber mesh implants showed a relatively high pull-out force in subcutaneous tissue (12.33 ± 5.29 N, mean \pm SD) and in muscle tissue ($2.46 + 1.33$ N).²⁹ Bobyn JD *et al.*²⁷, installed porous metal plates in the subcutaneous tissue of mongrel dogs. The largest metal pore size with the approximate range of 50-200 microns produced a mean peel strength of attachment of 27.5 g/mm after 16 weeks of implantation period. All these high values can be explained by the nature of the implant material used. A highly porous material will allow a better penetration of the soft tissues compared with the current materials.

The histological analysis showed a lack of direct contact between the soft tissue and implant surface. The observed formation of a fibrous capsule around the machined surface titanium implant with the presence of none or very minimal inflammatory cells, is similar to a previous study.³ Titanium is an 'inert' material and causes a minimal immune response and foreign body reaction in soft tissue.³⁰ This is the reason that many commercially available implantable devices (like pacemaker) are made of medical grade titanium (alloy).

In the present study, coated and non-coated PCL plate was found to be superior compared to the Ti implant in terms of fibrous capsule thickness. However, the fibrous capsule was found to be less mature and contained more macrophages and inflammatory cells at the tissue-implant interface than the Ti implants. The surface

modification with a CHA coating on PCL plate increased the surface roughness as shown in the SEM images (Fig. 3). PCL implant with CHA surface coating showed a significant improvement in capsule quality and tissue–implant interface quality, as observed by the reduction of inflammatory cells. This effect can be due to the increased surface roughness as created by the CHA coating. On the other hand, coating of PCL with CHA can also change the mechanical properties of PCL, i.e. the material becomes less flexible, which can also affect the soft tissue response.

PCL matrices are known to degrade at low rates by hydrolysis of the ester bonds and break down to their constituent monomer-hydroxycaproic acid, which then undergoes phagocytosis. PCL is characterized by a very low hydrolysis rate, which can vary from months to years.³¹ Therefore, the degradation process of PCL during the 5 weeks implantation period in this study was supposed to have no effect on the study result. For PCL coated with CHA, the CHA was still found to be intact at the end of 5 weeks and was clearly visible in the histological sections. Nevertheless, future research has to elucidate the effect of CHA on the biodegradation rate and bone regeneration properties of PCL.

Conclusions

The data of the current study indicate that none of the materials as well as surface modifications resulted in a superior soft tissue response. The peel test showed that adhesion of the soft tissues did not occur. Although, both types of PCL implants showed less migrational behavior compared with the Ti implants, soft tissue adhesion was not observed for any of the investigated implants, as demonstrated by the peel test data. Fibrous capsule formation around the non-coated and CHA coated PCL implants was less than around the Ti implants. On the other hand, an increased amount of interfacial inflammatory cells was present for all PCL implants compared with the Ti implants.

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MANDIBULAR RECONSTRUCTION WITH A BIOACTIVE-COATED CEMENTLESS Ti6AL4V MODULAR ENDOPROSTHESIS IN *MACACA FASCICULARIS*

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Introduction

In orthopaedics, the use of a metallic endoprosthesis for skeletal reconstruction after segmental bone resection has been reported for several decades.^{1,2} A modular concept was introduced in the late 1980s, which helps to eliminate the need for device customization.³ The prefabricated components of the various modular sizes allow to be assembled together during surgery.⁴ Both cemented and non-cemented methods have been used to fixate the device into the remaining bone. The cementless approach requires the use of materials that support the achievement of a good secondary fixation into the bone in order to avoid later loosening. Controversial failure and success data have been reported with the use of cementless orthopaedic devices.⁵ Abraham et al. found that the complication rate in the short-term outcomes of cementless modular endoprostheses to reconstruct the proximal femur, distal femur, and proximal tibia were relatively low to previously reported results of cemented implants.⁶

In oral and maxillofacial reconstruction, a titanium-6 aluminum-4 vanadium (Ti6Al4V) modular endoprosthesis was introduced for mandibular segmental reconstruction by Lee et al.⁷ The devices were fixed in the mandible of *Macaca fascicularis* using polymethyl methacrylate (PMMA) cement into the remaining bone stumps for 6 months. The study showed an abundance of bone formation around the body of the modular endoprosthesis. Problems encountered, however, included loosening of the module connections and infection. In addition, the soft tissue healing was not ideal resulting in dehiscence and hardware exposure in some cases, while uneventful healing was experienced with a developed ramus/condyle replacement in a later study.⁸⁻¹⁰

Based on the results of these previous studies, our research group modified the design of the endoprosthesis to better withstand the stresses from mastication forces. Therefore, the inter-modular connection of the device was re-designed using mechanical testing and finite element analysis to prevent component loosening.^{11,12} The biomechanical stability was found to be firm under simulated functional forces without having excessive stresses beyond the material strength of bone or titanium alloy.¹⁰

Further, the surface of the modular stems was modified to allow fixation into the mandibular bone without using bone cement, *i.e.* a cementless endoprosthesis. The bioactive surface coating with hydroxyapatite (HA) $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ and bioglass (BG) $[\text{SiO}_2\text{-Na}_2\text{O-CaO-P}_2\text{O}_5]$ are selected for titanium surface modification to improve soft and hard tissue healing. HA and related calcium phosphates (CaP)

are present in large amounts in bone.¹³ HA-based materials are basically not biodegradable, but are found to possess excellent bone biocompatibility¹⁴⁻¹⁶ and are widely used in clinical practice in both the orthopaedics and dental field. HA coatings on dental implants have been shown to accelerate bone apposition, thereby shortening the waiting period for implant restoration.¹⁷ The additionally provided surface roughness increases the interface strength even further.¹⁸ As a consequence, higher survival rates have been reported than for just commercial pure-titanium and titanium-alloy implants.¹⁹

Bioglass (BG) is an inorganic component with high bioactivity index and has the ability to bond to both soft as well as hard tissues.²⁰ BG has been shown to increase the expression of vascular endothelial growth factor (VEGF) *in vitro* and to enhance vascularisation *in vivo*, suggesting that BG might stimulate neo-vascularisation.^{21, 22}

The current study was designed to evaluate the effectiveness of bioactive-coated cementless modular mandibular endoprosthesis for mandibular reconstruction in *Macaca fascicularis*. The device was coated with HA/BG at the modular body surface, while the stems were coated with HA. We hypothesize that the bioactive-coated cementless mandibular endoprosthesis will have: (1) sufficient load-bearing capability for masticatory function, (2) good bone and soft tissue healing at the reconstruction site and (3) no loosening of the device components during 6 months of study period.

Materials and Methods

Animals

Nine, adult male, *M. fascicularis* monkeys, with an age of 4-6 years and weight of 6-7 kg were used in this study. All monkeys were pathogen free, presented with full adult dentition and were in healthy condition. The experimental protocol was approved by the Institutional Animal Care and Use Committee of SingHealth in Singapore. The animal laboratory has been certified by the International Association for the Assessment of Laboratory Animal Care (IACUC), Singapore.

Ti6Al4V Modular Endoprosthesis

Design and Characterization

The bioactive-coated cementless Ti6Al4V modular endoprosthesis is composed of two modular components, *i.e.*; (1) anterior module and (2) posterior module. The

connected device was 15 mm long, which was appropriate to replace and maintain the defect dimension. The device height was kept at 12 mm or 70% height of original mandible, while the width was 6 mm at occlusal and 4 mm at lower border, similar to the original size of mandible. The two modules were joined together with a dovetail connection and secured with a vertical screw. The anterior and posterior stems consisted of tapered screws of 12 mm length. The stems were non-self cutting and therefore drilling of the cancellous bone of the mandibular stumps was required before their insertion (Fig. 1).

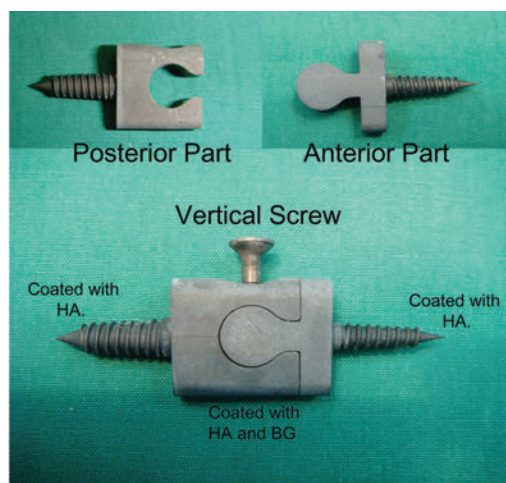


Figure 1. The bioactive-coated cementless Ti6Al4V mandibular endoprosthesis device. Body of the module was coated with Hydroxyapatite (HA) and Bioglass (BG). Both stem of the module were coated with Hydroxyapatite (HA).

Device surface coating with Hydroxyapatite (HA) & Hydroxyapatite/Bioglass (HA/BG)
The Ti6Al4V mandibular endoprosthesis was Al₂O₃ grit-blasted (60 mesh). Hydroxyapatite granules (HA, particle size 0.5-1.0 mm, CAMCERAM®, CAM Implants B.V, Leiden, Netherlands) and melt-derived bioglass crushed particles (Bioglass S53P4, particle size 30-315 µm, BonAlive®, Vivoxid Ltd, Turku, Finland) were used for coating deposition on the implant surfaces.

Hydroxyapatite (HA) and Hydroxyapatite/Bioglass (HA/BG) coatings were made by using a commercially available RF magnetron sputter deposition system (Edwards ESM100, Sussex, UK). The target materials for the coating deposition were the hydroxyapatite granules and bioglass particles. The endoprostheses were mounted on a rotating water cooled substrate holder. The distance with the targets was 80mm. During deposition, the argon pressure was kept at 5×10^{-3} mbar. The

following coatings were created:

- 1) **Endoprosthesis body modules** coated with a mixture HA/BG; at a discharge power of respectively 100W and 100W, with a deposition time of 20 hours, resulting in a coating with a thickness of 2 μm .
- 2) **Endoprosthesis stems** coated with HA; at a discharge power of 400W for both targets, with a deposition time of 5 hours, resulting in a coating with a thickness of 2 μm .

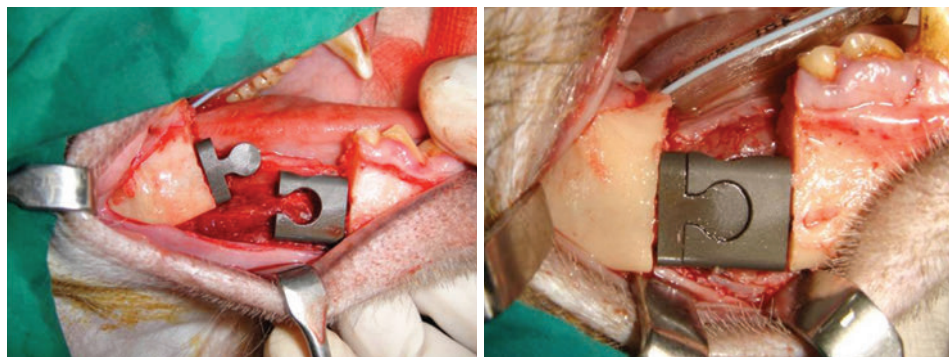
After deposition, the coated specimens were subjected to an additional heat-treatment for 2 h at 650°C. The composition of the coatings was confirmed by X-ray diffraction and Fourier Transform Infrared analysis. The devices were sterilized by autoclave before placement in the experimental animals.

Surgical Procedure

The animals were made to fast overnight. They received 0.05 mg/kg of subcutaneously atropine and 10 mg/kg of Ketamine preoperatively. Induction and maintenance of anesthesia were performed by a veterinarian using 2% isoflurane. Endotracheal intubation was done using oral endotracheal tubes of gauge 3.5 mm that was secured around the upper premolar tooth with ligature wire. Antibiotics (Ampicillin/Cloxacillin (Betamox®, Norbrook Pharmaceuticals Worldwide, Newry, Northern Ireland) 6-8 mg/kg subcutaneous) were given on induction and analgesics was given at the end of the surgery.

The operation site was disinfected with 1% cetrimide followed by 2% chlorhexidine and povidone iodine and draped for surgery. Using an intra-oral approach, two vertical incisions were made; between the second bicuspid and the first molar as well as behind the second molar. A horizontal incision 2-3 mm below the attached gingiva was made connecting the two vertical incisions. The periosteum was reflected to expose the lower border of the mandible. A tapered fissure bur was employed to perform the resection which included a 1.5 cm mandibular segment, containing the first and second permanent molars and the attached gingiva. Bleeding from inferior mandibular canal was easily staunched. The anterior and posterior bone stumps were prepared with a 0.8 mm fissure bur to 10 mm depth of the cancellous bone. The device stems were inserted with manual rotation until they fitted tightly and the edge of the module body was flushed with bone margin of the mandibular stump. The size of modular stems was selected to match the size

of mandibular bone stumps in each mandible. The modules were then connected and secured with the vertical screw at the superior aspect of the device (Fig. 2).



(2A)

(2B)

Figure 2. Mandibular segmental reconstruction using a bioactive-coated cementless Ti6Al4V modular endoprosthesis was inserted in segmental defect of *Macaca fascicularis* Monkey. Anterior and posterior modules were inserted tight fit to mandibular stumps (2A). The modules parts were connected and found immediate stability with well maintained the dimension of mandible (2B).

After insertion of the endoprostheses, the occlusion was evaluated. As intermaxillary fixation is not possible in this animal study, one titanium mini-plate (Medicon® miniplate, Germany) was fixed to the mandibular stumps using four 5mm screws. The buccinator and mylohyoid muscles were dissected, mobilized and approximated over the device using 4-0 Vicryl® sutures. This was followed by closure of the mucosa also using 4-0 Vicryl® sutures, therefore achieving a 2-layer closure.

Immediately after the surgery, a lateral mandibular radiograph was taken using a Siemens POLIMOBIL plus® machine (Siemens Medical Solutions, Erlangen, Germany) set at 40 kV for 2 ms at a distance of 70 cm. Thereafter, the animals were maintained in individual cages without restraint. Soft diet was provided until sacrifice. Ampicillin/Cloxacillin (Betamox®, Norbrook Pharmaceuticals Worldwide, Newry, Northern Ireland) 6-8mg/kg IM was given for 7 days post-surgically and repeated for 7 days, if there were signs of infection. Ketorolac trometamol (Toradol®, Hoffmann-La Roche Inc, Basel, Switzerland) 15-30mg/kg IM was given for 2-3 days post-surgically. During the experimental period, the animals were monitored regularly and lateral mandibular radiographs were repeated using the same protocol, at 1 month, 3 months and 6 months.

Specimen Retrieval

All animals were euthanized at 6 months postoperatively and weighed before euthanasia. The reconstruction site was examined for signs of infection, dehiscence, stability and occlusion. Mandible specimens were harvested from condyle to condyle with the device *in situ*. Soft tissue was removed except around the reconstructed site.

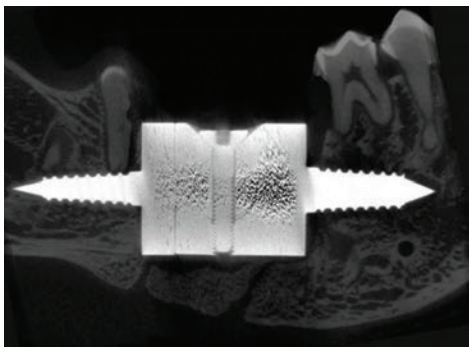
In four animals, the mandibles were harvested fresh as they were used for mechanical testing. In these animals, 3 ml of pentobarbitone sodium (Valabarb, Jurox, Rutherford, NSW, Australia) was injected into the cardiac chamber to euthanize the animals. The retrieved specimens were kept at frozen condition at -20°C until ready for analysis.

For the remaining five monkeys, harvesting was done after perfusion fixation. In these five animals, a 16-gauge intravenous catheter was inserted into the left ventricle and used to rinse the circulation with 300-500 ml of Hartman's solution followed by 750 ml of a mixture of 2.5% paraformaldehyde and 2% glutaraldehyde. The specimens were kept immersed in 10% glutaraldehyde.

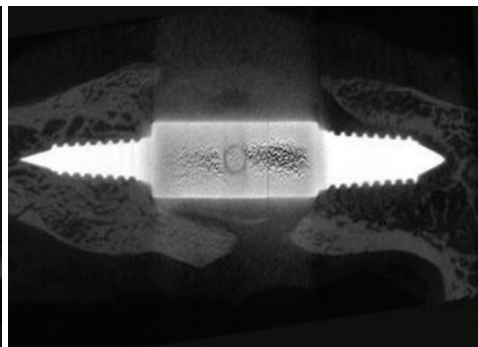
Analysis

Micro-CT Evaluation

The specimens containing the endoprosthesis were scanned using a GE eXplore Locus SP MicroCT scanner (GE Healthcare, Thermo Scientific, Waltham, MA, USA), with a focal spot of $8\text{ }\mu\text{m}$ and pixel size of $18\text{ }\mu\text{m}$ and scanning configuration isotropic voxels of $8\text{ }\mu\text{m} \times 8\text{ }\mu\text{m} \times 8\text{ }\mu\text{m}$ focal spot size and isotropic resolutions at $8\text{ }\mu\text{m}$ (Fig. 3).



(3A)



(3B)

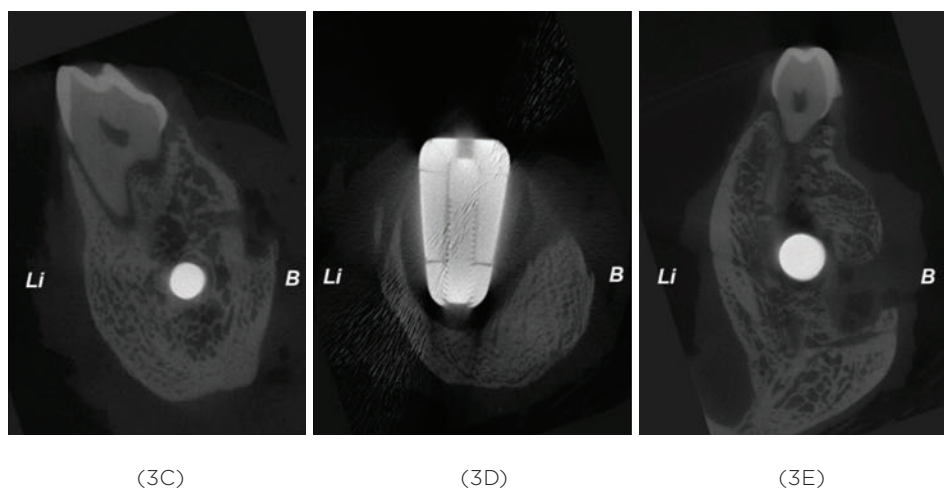


Figure 3. Micro CT scan image; A) lateral view, B) occlusal view and cross sectional views, C) anterior stem, D) mid body and E) posterior stem.[B; Buccal and Li; Lingual]

The mini-plates and screws were removed before the scanning process to avoid scattering. The scan area was extended to the maximum diameter of scan view and covered beyond the region of interest (ROI) at the reconstruction site, including the entire anterior and posterior stems. The digitized signals were then transferred to a computer for reconstruction of the micro-CT slices. Standardized calibration was used compared to bone, air and water. All images were calibrated in Hounsfield units (HU) for quantitative analysis. New bone was analyzed using MicroView® 2.2 software (GE Healthcare, Waukesha, WI, USA).

Micro-CT slices were reconstructed perpendicular to the long axis of the mandibular reconstruction. The percent bone volume (BV %) around the stems of the device was analyzed modified from the method described in a previous study.³ Briefly, three circular regions of interest (ROI) with standardized diameter of 0.52 mm were selected for each specimen. These were located directly next to stem surface in the buccal, lingual and inferior aspects. The selection of an ROI superior to the stem was not done as a tooth was frequently present in that area. In the case where a screw hole or tooth structure was seen to be within the ROI, the ROI was manually moved to the immediate adjacent area. Percent bone volume (BV %) was calculated using MicroView™ 2.2 GE Healthcare computer software (Fig. 4).

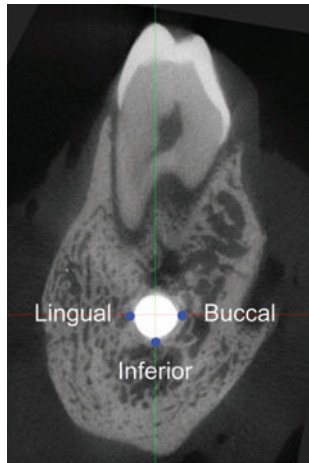


Figure 4. The area of interest of bone growth around the Ti device's stem. A standardized diameter of 0.52 mm in 3 areas was selected for each specimen. There was located directly next to the stem surface at buccal, lingual and inferior region. Percent bone volume (BV %) in ROI was identified and analyzed using Microview computer software.

Mechanical Testing

The four specimens for mechanical testing were processed at the Biomedical Engineering Laboratory, College of Engineering, The University of Michigan, Ann Arbor, MI, USA. Three-point bending test was done to determine their stiffness using an MTS Alliance RT/30 Elite™ Controller testing machine (TestResources Inc, Minnesota, USA).

The specimens were thawed from -20°C to room temperature for 2 hours before mechanical testing. All specimens were maintained in a moist condition until the test was completed. Before mechanical testing, the mini plates and screws as well as most of the soft tissue around the mandible were removed. Due to the instability of the bone segments of the samples, a thin layer of muscle tissue enveloping the mandibles was preserved to help maintain the integrity of the reconstruction site. Bilateral mandibular coronoid processes and canine cusps were trimmed to prevent interference with the fixation jig during mechanical testing. Subsequently, each specimen was placed on the biomechanical 3-point bending testing fixtures in a reverse position with both condyles and mid-anterior lingual bone surface placed on the custom-made jig. A force with constant displacement rate of 25 mm/min was applied on the lower border of the mandibular until 111.20 N was reached. The load-displacement data were recorded at a frequency of 15 Hz to determine the elastic stiffness (N/mm) of the reconstructed mandibles without breaking

the specimens. After mechanical testing, all specimens were immersed in 10% formaldehyde for histological analysis.

Histological Analysis

The specimens were reduced in size, dehydrated in a graded series of ethanol, embedded in methyl methacrylate resin and polymerized. The tissue blocks were mounted in a modified inner circular saw microtome (Leica® RM 2165, Wetzlar Germany) and 10 µm thick sections were prepared. Serial bucco-lingual cross sections were stained with methylene blue and basic fuchsin for histological and histomorphological analysis. At least, seven bucco-lingual histological cross sections were made from the reconstructed mandible from each specimen *i.e.* one at the midline of the device's body, three at the junction between device body and the stem and three at mid of the scaffold's stem.

Histological sections were recorded using a light microscope equipped with a camera (Carl Zeiss, Oberkachen, Germany) and then evaluated with a digital image analysis system (Leica QWin Pro, Wetzlar, Germany). Histological and histomorphometrical analysis was done in three regions; (1) mid scaffold, (2) junctions between device body-stem and (3) mid posterior stem. Bone quality and quantity analysis was performed using a bone score scale for 'bone presentation' and 'bone-device contact' at each region (Table 1). The percentage bone–stem contact and the BV% were analyzed for the area around the posterior stem.

Table 1. Parameters and scoring for histology evaluation by light microscopy

Parameter	Score
<ul style="list-style-type: none"> Bone Formation around device (Body of device, Body-Stem Junction, Stem) 	2 : Completely surrounded with bone 1 : Partially surrounded with bone 0 : No bone (fibrous formation)
<ul style="list-style-type: none"> Bone contact at device surface (Body of device, Body-Stem Junction, Stem) 	2 : Completely device surface contact with bone 1 : Partially surface contact with bone 0 : No bone contact (fibrous formation)
<ul style="list-style-type: none"> Percent bone-device surface contact (%) (Stem) 	0-100%
<ul style="list-style-type: none"> Bone volume and Percent Bone Volume (%) (Stem) 	Bone volume (mm ³) and Percent Bone volume (0-100%) at the Area of bone at 2 mm from stem surface

Statistical Analysis

Descriptive statistic analysis was applied for mechanical test result using stiffness values due to limited in sample size.

Micro CT and histological measurements were statistically analyzed using SAS 9.2 statistical software (SAS Institute Inc, Cary, North Carolina). Measurements were evaluated by non-parametric Mann-Whitney U Test to identify the existence of differences between the anterior and posterior stem. The results were also compared with those of a previous study on PMMA cemented endoprosthesis.²³ A *P*-value < 0.05 was considered statistically significant.

Results

Clinical Observation

The operation was completed in an average time of 1.5 hour for each animal. The marrow cavity preparation was performed without difficulty and the modular stems were able to fit tightly into the native marrow bone quite easily by manual manipulation. All animals recovered well after operation.

At one month post surgery, five monkeys presented with good wound healing, while three monkeys showed a small fistula and one animal had wound dehiscence related to loosening of the mini-plate and screws. This loosened mini-plate and screws were removed and wound was debrided and re-sutured. None of the monkey showed signs of severe infection or sepsis. The occlusion and bone segments appeared stable. The lateral mandibular radiograph showed that the devices were in good position.

At three months, wound dehiscence was noted at the superior aspect of the endoprosthesis body in five animals, including the one that had dehiscence at 1 month. Oral fistulas were found in another two animals, while healing proceeded well in the remaining two animals. The occlusion and bone segments in all animals remained stable and none showed any signs of severe infection or sepsis. Lateral radiographs indicated that enhanced bone formation had occurred at the lower border of the defect in eight animals.

At 6 months, all monkeys were still healthy and there was no obvious weight loss. Two of nine reconstruction sites remained stable with good wound healing (Fig. 5A). One animal, which previously presented with a small wound dehiscence, was found to have healed well. The remaining six monkeys presented with exposure of the superior surface of the device modules (Fig. 5B). In two of these,

the fixation screws were loose and consequently, the mini-plates were displaced. The reconstruction site and occlusion remained stable in all animals. Radiographs showed increased amount of bone formation at the reconstruction sites especially at the lower border and lingual site (Fig. 5C and D).

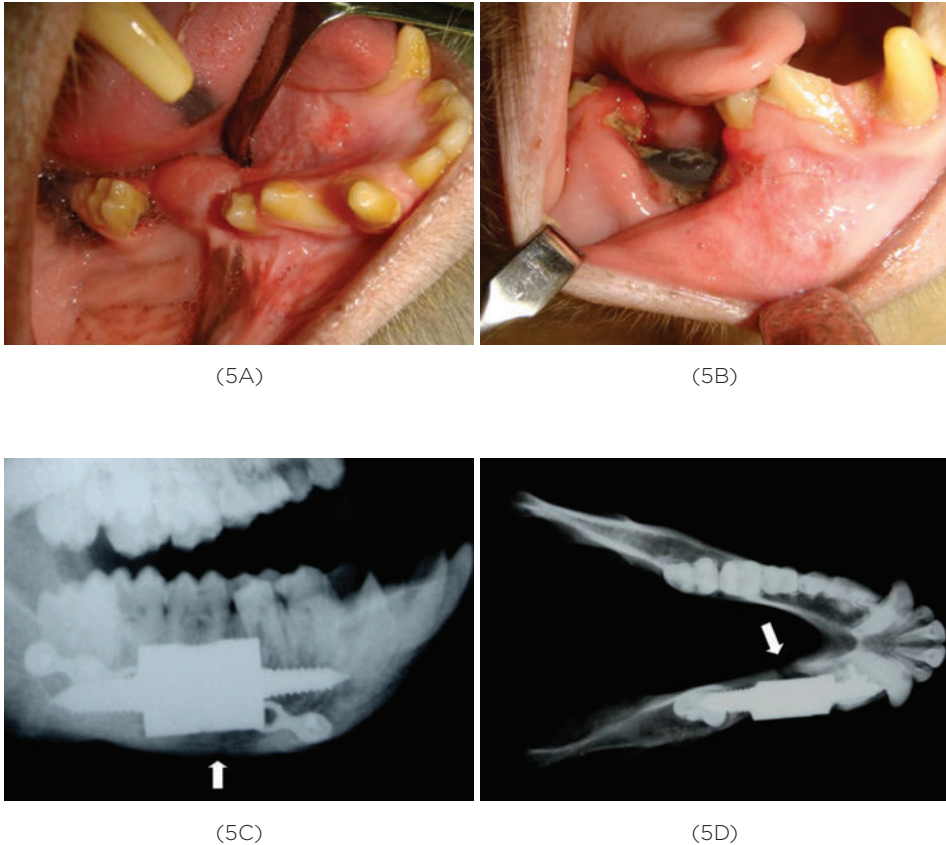


Figure 5. The clinical and radiographic pictures at 6 months post operation. The reconstruction sites remained stable and intact in 3 animals (A), however the remaining 6 animal presented with exposure at occlusal surface of the device (B). Radiographs showed bone formation at the lower border and lingual side of the reconstruction site (arrows). Mandibles specimens were maintained in shape and contour (C) and (D).

Micro-CT Result

The micro CT scans generally showed that the endoprosthesis had maintained the contour and dimension of the reconstructed side of the mandible, which was grossly similar to the contra lateral non-operated side. All specimens showed a variable amount of bone formed at the lower border and lingual aspect of the defect but none had complete bone bridging between segments.

Micro CT analysis indicated a mean BV% of $55.62 \pm 32.84\%$ at the buccal, $56.19 \pm 27.60\%$ at the lingual and $59.98 \pm 39.42\%$ at the inferior regions of the **anterior stem** compared to $77.18 \pm 30.73\%$ at the buccal, $75.27 \pm 35.00\%$ at the lingual and $63.33 \pm 21.72\%$ at the inferior regions of the **posterior stem**. There was no significant difference in percentage bone volume between the anterior and posterior stem in all regions (Buccal; $p=0.31$, Lingual; $p=0.31$ and Inferior; $p=0.86$ (Fig. 6).

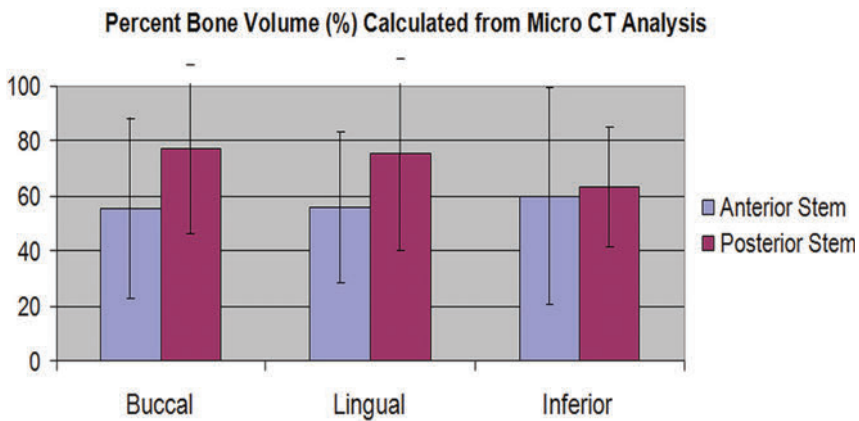


Figure 6. Bone volume (BV %) around the stem of the device, three circular regions of interest (ROI) with standardized diameter of 0.52 mm measured directly next to stem surface

Mechanical Testing

The four mechanically tested specimens showed a mean stiffness of 110.43 ± 59.53 N/mm when loading force was applied to the reconstruction side and 164.95 ± 172.44 N/mm when loading force was applied to the contralateral side of mandible (Fig. 7).

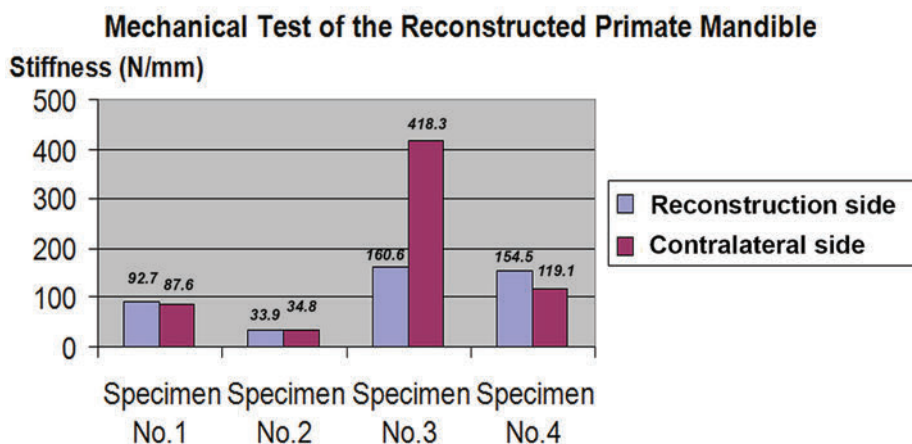


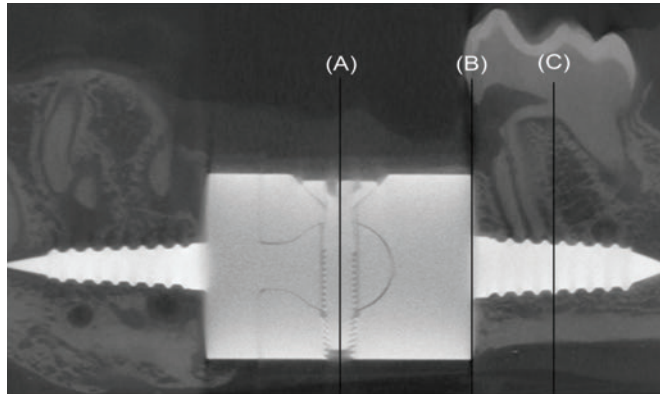
Figure 7. Stiffness values from 3 point bending

Histological and Histomorphological Analysis

Light microscopical analysis

The histological sections through the midline of the prosthesis module showed a thick fibrous capsule and minimal inflammatory cells surrounding the metallic device. Newly deposited mature bone was found in most of the specimens (8 of 9) at the inferior border without direct bone contact to the body of the modular surface. Discontinuity of the covering soft tissue seen on occlusal and buccal surfaces correlated with the clinical finding of wound dehiscence (Fig. 8A).

At the junction between body-stem of the device, bone had formed, which showed direct bone contact in 4 of 9 specimens (Fig. 8B). The sections through the posterior stems showed that the stems were surrounded by native mandibular bone in 7 of 9 specimens (Fig. 8C), while the remaining two specimens presented a buccal fenestration. Four of the specimens showed excellent bone-stem contact, while in the remaining five specimens the stem surface was surrounded by a thick fibrous capsule showing varying degrees of inflammatory reaction (Fig. 9).



Histology section regions

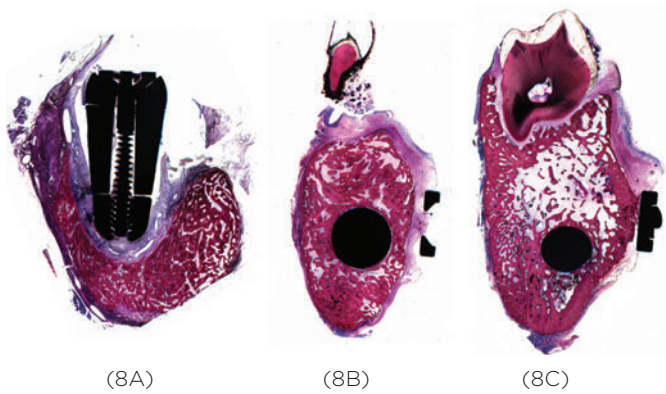


Figure 8. The 2D-image taken from Micro CT scan shows region where histological sections were made. Histology sections (magnification 1X) were made at the mid prosthesis device's body (A), junction of device body-posterior stem (B) and section of posterior stem (C).

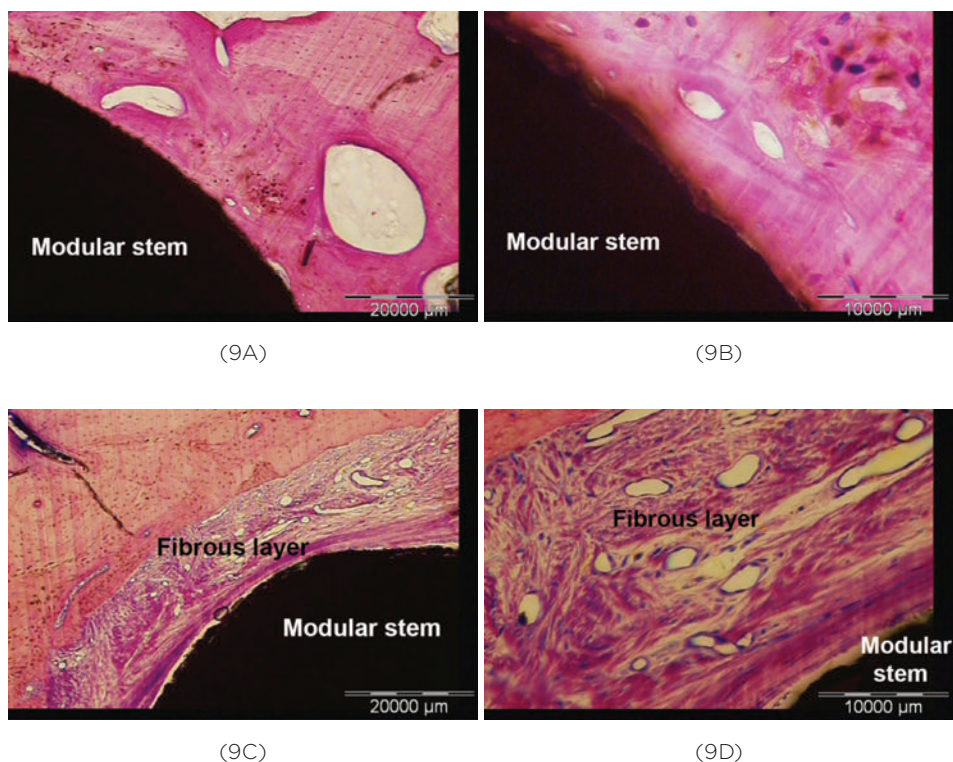


Figure 9. The histology section show excellent ‘device-bone interface’ at the posterior stem area which coated with HA; (A) Magnification10X and (B) Magnification 40X. Fibrous capsule with inflammatory cells was found at the stem section in some specimens; (C) Magnification10X and (D) Magnification 40X.

Histomorphological analysis

The mean bone score scale for bone presentation around the devices (range from 0 - 2)’ was 0.22 (buccal) and 0.89 (lingual) at the **mid section of the device body** and 0.81 (buccal) and 1.56 (lingual side) at the **junction of the device body and the stem**, while the mean bone score around the **stem** was 1.78.

The mean grading scores for bone-device contact (range from 0 - 2) was 0 or no bone contact at the **mid section of the device body** in all samples, 0.56 (buccal) and 0.63 (lingual) at the **junction of the device body and the stem** and 0.52 around the posterior **stem** of the device. The percent bone-stem contact around the posterior stem was analyzed for 4 of 9 specimens, which had score scale of at least 1, and was found to be 64.17% (44.30% - 80.17%).

The mean BV (%) was calculated from the area of 2 mm around the surface of stem device (n=9) and was found to be 45.56% (range from 21.10%-66.50%).

Discussion

A modular endoprosthesis has been used with great success in orthopaedic surgery.²⁴ The major anatomic joints with their adjacent segmental bone can be reconstructed safely and reliably with a modular endoprosthetic replacement.²⁵ The cementless endoprosthesis was designed to prevent aseptic loosening of the endoprosthesis. In theory, a better stability can be achieved by having direct bone contact with the device at the interface in a cementless endoprosthesis, while this is nearly always absent when bone cement is used.²⁶ The cemented modular endoprosthesis for mandibular reconstruction, as used in an earlier monkey study, was found to result in good function and stability of the reconstruction sites up to 6 months. Nevertheless, fistulas were noted at the reconstruction site that seemed to be related to loose intermodular connection screws²³. In the effort to overcome this problem, we developed the bioactive-coated cementless modular endoprosthesis as used in the current study.

The modular parts of the endoprosthesis were designed connection as a dove-tailed interlock. Our observations revealed that this design prevented loosening of the intermodular connection components throughout the 6 months study period in all animals. This clinical observation corroborated with the results of the *in vitro* biomechanical study reported earlier by Wong *et al.*¹¹ Radiographs and micro CT scans in this current result confirmed that the dovetail locking mechanism and vertical pin remained in a stable position throughout the experimental period. Therefore, it can be deduced that this new dove-tail interlock design is able to solve the problem of inter-modular fixation screw loosening.

The stems of the current endoprosthesis were designed as tapering screws that were manually inserted into the marrow space of the mandibular bone without using bone cement. The surgical technique was simple and shortened the operation time. The tapered stem design allows matching of the stem part with the size of the individual mandibular bone stump. As a consequence, the reconstructed mandible provided an immediate accurate three-dimensional replacement of the lost part of the mandible with initial stability.

Although titanium is commonly used as a favourable bone implant material, a number of studies have modified their titanium implant surface to improve the bioactive properties. For decades, studies of hydroxyapatite coating on the titanium implant surface reported a significant improvement of direct bone-implant contact.²⁷⁻³⁰ Therefore, HA coating deposition on the stem device surfaces was selected in this study. The result showed positive effect of HA coating with

good bone contact at stem–bone interface from the micro-CT and histological analysis.

Besides bone-device contact, good soft tissue healing is essential to the success of the endoprosthesis reconstruction. To enhance the soft tissue-titanium implant interface in medical and dental uses, changing the implant surface topography increases the survival rate of devices by improving soft tissue stability to help seal against bacterial leakage.³¹ Lee *et al.* reported that HA/BG coated submucosal plates showed favourable oral mucosa adaptation at 1 month after implantation.³² They found a thinner capsule quantity and an increased capsule quality and interface quality score for HA/BG coated implant compared to acid etched Osseotite® surface plates.³² However, HA/BG coating showed no beneficial effect on soft tissue healing in the current study. The height of the device, a limited blood supply and a lack of a thick muscle protection layer and the lack of stability of the reconstructed site during the healing period all contributed to an unfavourable soft tissue response, which apparently cannot be overcome by a HA/BG coating.

The results showed that only three of the nine animals had a complete soft tissue healing with a stable reconstruction system. In contrast, the remaining six animals suffered from complications of intraoral wound breakdown, similar to those mandibular reconstruction using an over contour conventional titanium plates with or in patients with radiated tissue. Local infection was found to be related to loosening of the mini-plate and screws, subsequent loss of rigid fixation of the device stem(s). The monkeys were not able to be placed into intermaxillary fixation due to the requirement for feeding, which placed additional strain on the device stems during masticatory function, which could easily be avoided in humans. It was also observed that the monkeys frequently inserted their fingers into their oral cavities. This was probably due to a feeling of discomfort, and likely disturbed normal wound healing as well.

The cementless, stem retained endoprosthesis requires sufficient stability in bone at the initial stages of healing, to provide an optimal environment for osseointegration. The current method of fixation with a semi-rigid plate does not provide for sufficient stability, resulting in non-union of the device stems to the surrounding bone. Further mechanical testing of the stability requirements of the endoprostheses will provide more data with which to design a proper fixation system to improve the stability of the device during the early phases of stem osseointegration.

In addition, an extraoral approach instead of an intraoral approach for the endoprosthesis installation possibly prevents early exposure of the device to the

non-sterile oral cavity and can reduce wound breakdown and infection.

On basis of these findings, we have to reject the hypothesis that: (1) the current designed cementless mandibular endoprosthesis has sufficient load-bearing capability and (2) a HA/BG titanium surface is able to achieve soft tissue attachment and healing.

Henderson *et al.* described that the causes of failure in the orthopaedic endoprosthesis that could relate to 5 reasons; soft-tissue failures (Type 1), aseptic loosening (Type 2), structural failures (Type 3), infection (Type 4), and tumour progression (Type 5).³³ In the *in vitro* study reported by Wong *et al.*, on the mechanical testing and finite element model of the modular endoprosthesis, the weak point of the device was found to be at the superior surface of the stems, especially at the junction between the stem and the body of the device.¹¹ The irreversible deformation described as bending or crack lines, was found to be located at the junction of the body and the device's stem, related to high shear stress. However, this weak point was not found to be present in our design, as described in the current animal study. Our postulation of the laboratory difference in the mechanical finding reported by Wong *et al.*, in this current *in vivo* study could be due to a few reasons; (1) the design of the taper stem used in this study was stronger than the cylinder screw used in the laboratory, (2) the porosity structure and the biologic factors of the mandible used in the animal model differ from those tested in the artificial mandibles. For example, in the *in vivo* study there was the presence of a natural bone structure with cells and blood supply, which contributed to a healing process and the presence of masticatory function and loading in the animals, and (3) an additional mini-plate with screws was inserted in the animal study, but not in the *in vitro* study. This was included to increase the stability of the reconstruction site as intermaxillary fixation (IMF) was not possible in the animal model. The mini-plate fixation was believed to act as a cross-brace to minimize movement of the bone segments and the device during masticatory function so as to allow a period of healing to achieve bone contact with the stem surface. The mini-plate used could also contribute to the sharing of the loading force, yet this additional fixation had not been tested and analyzed in the laboratory.

Mechanical analysis of the reconstructed specimens has often been used to assess the performance of the prosthesis and the bone-device interface.³⁴ The test methodologies that mimic a simulating bite force for mandibular reconstruction have been reported in the literatures.³⁵⁻³⁷ The current mechanical test setting was modified from a previous study by Schupp *et al.* on osteosynthesis systems in segmental resection of on the synthetic mandibles.³¹ However, we subjected our

specimens to mechanical testing without fracturing them, which allows subsequent histological analysis of the specimens. To the best of the authors' knowledge, the stiffness value of a monkey mandibular bone has not been reported in the literature. Also, no control data are available either from intact fresh specimen or previous PMMA cemented mandibular endoprosthesis study.²³

Prior to mechanical testing, a pilot study was done using a dry intact macaque mandible (with the limitation that no fresh specimens were available) to determine the maximum load before fracture of the mandible occurred. A force of 889.6 N had to be applied to achieve fracturing. Subsequently, the mandibular specimens were tested using the same conditions, with the exception that the maximum load was reduced to 12.5% of the failure load to avoid fracturing of the specimen. Loading force was applied on both the operated and non-operated site, although it has to be noted that it is possible that the stiffness value of the non-operated side was affected by the reconstructed side, as the specimens were fixated on both condyle heads and anterior mandible.

Further, we observed that the stiffness values of the reconstructed specimens showed a wide data distribution. This can be due to the lack of direct bone contact with the anterior stem. It is possible that the anterior part of the endoprosthesis received a higher functional loading force due to the long span segment of the opposite side of mandible.

BV % determined by micro-CT scan around the stem part of the endoprosthesis (Table 2) was found to be significantly higher at the buccal and lingual regions of the anterior stem as well as the buccal regions of the posterior stem when compared with previously reported data.²³ We suppose that this effect is due to the non-cemented approach used. As a consequence, the transfer of load proceeds directly to the surrounding bone without intervening mass. This can result in an increased bone deposition, as characterized by an enhanced BV%. There was no significant difference in BV% around the anterior stem compared with the posterior stem at buccal, lingual and anterior to the stem. A similar observation was made when the analysis was done with the ROI positioned 1.5 mm away from the stem surface (data not shown). Based on these Micro-CT data, the histological analysis was performed only at the posterior stem and not for the anterior stem region. Three of the nine specimens showed better soft tissue healing and a stable mandibular reconstruction, with a high percentage of bone–device contact. This improved healing can be due to the presence of a HA coating on the stem part. Also, the histological sections showed that the regenerated bone was abundantly present between the mandibular segments and around the body of the module. This observation was similar to

the results with the PMMA cemented mandibular endoprosthesis.²³ However, light microscopy indicated that the new bone around the body of the device had no direct contact with the device surface and an intervening fibrous tissue layer was seen. Probably, this is creeping bone formation or a reaction from an intact periosteum. This bone formation is expected to be limited in the massive tissue resection of a malignant tumour during ablative surgery, with the periosteum commonly removed.

Nevertheless, the sample size of specimens in the current study was limited and complicated with local infection and wound dehiscence, and therefore the results need to be interpreted with caution. There were also no control devices, *i.e.* endoprosthesis without additional HA coating or comparisons between different fixation method at the reconstruction site. Unfortunately, this was not possible due to the limitation in available animals.

Table 2. The comparison of bone volume (calculated from Micro CT Scan) with previous study

Group	Percent Bone Volume Around Stem of Device (<i>Mean ±SD.</i>) Calculated from Micro CT Scan					
	<i>Anterior stem</i>			<i>Posterior stem</i>		
	Buccal	Lingual	Inferior	Buccal	Lingual	Inferior
Previous study¥ Modular Endoprosthesis with PMMA cement [Number of Specimens(n)=4]	10.83 (±6.35)	14.59 (±7.47)	29.42 (±28.13)	38.45 (±29.68)	43.07 (±13.89)	45.89 (±31.79)
Current study Bioactive-coated cementless modular endoprosthesis : non- cemented [Number of Specimens(n)=9]	55.62 (±32.84)	56.19 (±27.60)	59.98 (±39.42)	77.18 (±30.73)	75.27 (±35.00)	63.33 (±21.72)
<i>P</i> -value	p=0.02*	p=0.05*	p=0.20	p=0.05*	p=0.20	p=0.50

NOTE: ¥ Data from Lee et al. [*Int. J Oral Maxillofac Surg.* 2009 Jan; 38(1):40- 7]

*Significant difference at $p < 0.05$

Conclusion

Based on the current findings, it can be concluded that the cementless modular endoprosthesis can potentially be used for the successful reconstruction of the mandible. The success of a cementless mandibular endoprosthesis is dependent on several factors, *i.e.* design of the device, device's material and surface coating, surgical technique and primary stability. In future studies, several modifications have to be tested to create an optimal device *i.e.* reduction of the height of the device's body, provision of sufficient stability during the early healing phase, and improvement of the soft tissue attachment to the titanium surface. In addition, the uses of an extra-oral surgical approach can possibly achieve a better result.

Competing interests: None declared

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Ethical approval: The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of SingHealth, Singapore (IACUC #2009/SHS/454).

Patient consent: Not required

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SEGMENTAL MANDIBULAR BONE RECONSTRUCTION WITH A CARBONATE-SUBSTITUTED HYDROXYAPATITE-COATED MODULAR ENDOPROSTHETIC POLY(ε-CAPROLACTONE) (PCL) SCAFFOLD IN *MACACA FASCICULARIS*

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Introduction

Mandibular bone provides the skeletal base for teeth, which play a critical role in mastication, speech, and maintenance of the facial profile.¹ Reconstruction of large mandibular defects caused by trauma, tumor resection, and congenital defects is a significant clinical challenge.² Various methods of mandibular defect reconstruction have been reported in several surgical techniques involving reconstruction plate, free bone graft, pedicle bone graft, particulate bone cancellous marrow graft, microvascular free flap, transport distraction osteogenesis, modular endoprosthesis, and tissue engineering. Each technique has its own benefits and limitations. The current 'gold standard' treatment is either an autogenous free bone graft or a vascularized microvascular free fibular flap.¹ However, even this standard has limitations in terms of aesthetic and functional outcomes when the bone graft does not replicate the original complex mandibular geometry. The procedure is also time-consuming, including a hospital stay, and is associated with significant donor-site morbidity. These problems have led clinicians to explore alternative procedures for mandibular reconstruction.

In 2006, Lee and co-workers³ introduced a modular endoprosthesis for mandibular reconstruction. This device was made of titanium alloy, and the prosthesis stems were cemented into the remaining bone stumps on either side of the mandibular defect. The experimental animal study with this approach showed an abundance of bone formation around the body of the modular endoprosthesis at 6 months post-surgery; however, the soft-tissue healing was not ideal, resulting in dehiscence and, in some cases, hardware exposure. In addition, hardware failure (i.e., several screws of the modular endoprosthesis becoming loose) was found.³⁻⁵

A recently introduced alternative direction for mandibular bone reconstruction involves tissue-engineering techniques, which offer potential advantages such as the absence of donor-site morbidity and an ability to regenerate original bone geometry. Based on the results of the earlier study with the titanium modular endoprosthesis, we decided to pursue such a tissue-engineering approach and designed a biodegradable osteoinductive modular endoprosthetic scaffold for the regeneration of a segmental mandibular defect.

Poly(ϵ -caprolactone) (PCL) was selected for the manufacture of the endoprosthesis. PCL is a bioresorbable polymer with potential applications for bone and cartilage repair and has several advantages over other polymers. PCL is more stable in ambient conditions, is readily available in large quantities, and can be easily combined as well as processed with other materials to further formulate

the tissue response.^{6,7} PCL scaffolds can be fabricated by selective laser sintering (SLS), a rapid prototyping/Solid Free-Form Fabrication (SFF) technique to fit complex anatomic locations. This technology allows for the design of a scaffold with computationally predicted properties and a possible global anatomic architecture that matches the original bone defect and supports the in-growth of bone tissue.^{8,9} The selected percentages of scaffold porosity, structure, and mechanical design can be controlled. Porosity between 37 and 55% has been reported to possess mechanical properties comparable with those of human trabecular bone, and the compressive modulus of such a scaffold was found to be within the 52- to 68-MPa range, with ultimate compressive strength within the 2.0- to 3.2-MPa range.⁹ This makes such a manufactured material an attractive substitute for human bone and enhances its application for bone regeneration.

Osteoinductive or autoinductive bone formation is a mechanism of cellular differentiation towards bone of one tissue due to the physicochemical effect or contact with another tissue.¹⁰ It generally can only be induced by heterotopic implantation of demineralised bone matrix (DBM) or BMPs into a region where bone does not naturally grow.¹¹ PCL is not osteoinductive, which limits its applications in the regeneration of critical-sized bone defects. To enhance the bone-regenerative properties, PCL scaffolds containing growth factors, and in conjunction with a carbonate-substituted hydroxyapatite coating, have been shown to be promising in creating osteoinductive scaffolds. In 2011, Suárez-González *et al.* reported mineral coatings on polycaprolactone scaffolds serving as templates for growth-factor binding and release.¹² Mineral coatings were formed by a biomimetic approach that consisted of the incubation of scaffolds in modified simulated body fluids (mSBF) with a composition similar to that of human plasma, but with double the concentrations of calcium and phosphate. Such scaffolds demonstrated the ability of attachment and sustained release of growth factors, such as VEGF and BMP-2, which were dependent on the solubility of the mineral coating.^{12, 13}

In addition to growth factors such as rhBMP-2¹⁴⁻¹⁸ and rhBMP-7,^{14, 19-21} bone marrow stromal cells (BMSCs) have been found to be a stimulus for bone regeneration, being capable of differentiation into mesenchymal tissues such as bone and cartilage.²²⁻²⁶ BMSCs or cultivated osteoprogenitor cells can be seeded into a porous scaffold, and, when given the appropriate environmental signals, can be directed down the osteogenic lineage and cued to form bone tissue.²⁷ BMSCs are readily available and can be isolated from bone marrow or fat tissue. Numerous successful animal studies have been reported, where mandibular continuity defects were regenerated on a scaffold provided with bone marrow stromal cells.^{26, 28-30}

In view of the above, the aim of the current study was to regenerate a segmental mandibular bone defect by means of a 3-D designed PCL scaffold provided with a carbonate-substituted hydroxyapatite (CHA) coating for the delivery of osteoinductive factors to the defect site. The study compared the use of empty PCL scaffolds (PCL-control), PCL scaffolds seeded with autologous bone marrow cells in a bovine collagen type I gel (PCL-CELL), and PCL scaffolds provided with additional rhBMP-2 (PCL-BMP). We hypothesized that the osteoinductive scaffold loaded with rhBMP-2 or bone marrow cells could achieve bone union and overlying soft-tissue healing with sufficient load-bearing capacity within 6 months after its installation into the mandibular segmental defect in a non-human primate model.

Materials and Methods

Animals

Twenty-four healthy, adult male *Macaca fascicularis* monkeys, with an average weight of 6-7 kg, were used in this study. The experimental protocol was approved by the Institutional Animal Care and Use Committee of SingHealth in Singapore. Animal surgery was performed at the SingHealth Experimental Animal Centre (SEMC), Singapore. The animal laboratory has been certified by the International Association for the Assessment of Laboratory Animal Care (IACUC), Singapore.

PCL Scaffolds and Calcium Phosphate Coating

Poly-ε-caprolactone (PCL) powder (CAPA 6501, Solvay Caprolactones, Warrington, Cheshire, UK) was used to fabricate the PCL scaffolds. This particular form of PCL has a melting point of 60°C, a molecular weight of 50,000 kDa, and particle size distribution in the 10- to 100-μm range.

Fabrication of PCL Scaffolds

The PCL scaffolds were fabricated by the Department of Biomedical Engineering, University of Michigan, Ann Arbor, USA. For the fabrication process, computed tomographic (CT) scans of dry monkey mandibles were made, and scaffolds were fabricated via laser sintering as previously reported.⁹ With the CT images as a guide, mandibular scaffolds were created with a controlled architecture. The design was then exported to a Sinterstation 2000™ machine (3D Systems, Valencia, CA, USA) in STL file format and used to construct scaffolds layer-by-layer, with a powder layer, by the selective laser sintering (SLS) processing. The body of the implant was

15 mm long and 12 mm high and possessed a 3-D orthogonal periodic porous architecture. The body of the implant was also modular, comprised of anterior and posterior components, which were fixed together with a special lock-on design. Mechanical analysis confirmed that the “dovetail joint” between the two components was failing (fracture point) at 30 N. The stems were 12 mm long, 4 mm high, and 2.5 mm in diameter and were made to fit the marrow space of the mandible as closely as possible. In cross-section, the stems showed a star-shaped appearance, to increase the bone-scaffold contact area. Micro-CT analysis reviewed that the PCL scaffolds had a reasonably homogenous structure, with total surface area of 2089 mm², porosity 75%, and pore size of 1200µm. All pores are interconnected (Fig. 1).

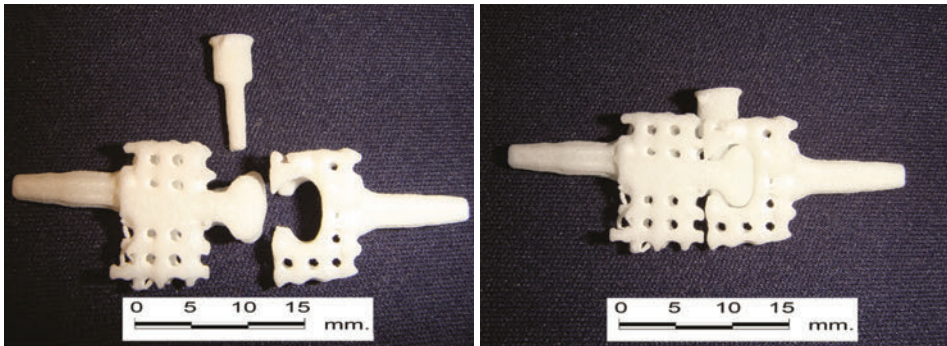


Figure 1. Poly(ϵ -caprolactone) (PCL) Polymer endoprosthesis mandibular scaffold has anterior and posterior parts with a ‘dovetail’ inter-lock design. A scaffold pin is used to stabilize the two parts. Anterior and posterior endoprosthesis stems are designed to fit into the prepared cavity in the cancellous bone of native mandibular segments. The surface of scaffold is coated with carbonate-substituted hydroxyapatite (CHA).

PCL Scaffolds with Carbonate-Substituted Hydroxyapatite (CHA) Coating

The PCL scaffolds were used as-received or provided with a carbonate-substituted hydroxyapatite (CHA) coating. The scaffold components were incubated in a modified simulated body fluid (mSBF) for 8 days at 37°C under continuous rotation. Prior to mSBF incubation, the PCL components were hydrolyzed in 1 M NaOH for 60 min. After hydrolysis, plates were rinsed and incubated in the mSBF. The mSBF solution had a composition similar to that of human plasma, but with double the concentrations of calcium and phosphate to enhance mineral growth, and was prepared as previously reported:^{9, 13} briefly, 141 mM NaCl, 4.0 mM KCl,

0.5 mM MgSO_4 , 1.0 mM MgCl_2 , 4.2 mM NaHCO_3 , 5.0 mM CaCl_2 , and 2.0 mM KH_2PO_4 in deionized ultra-filtered water, with pH adjusted to 6.8 with 2 N HCl or 2 N NaOH. Before use in the animal study, all scaffolds (non-coated and CHA-coated) were sterilized by ethylene oxide gas.

Bone Morphogenic Protein

Recombinant human Bone Morphogenic Protein-2 (rhBMP-2) in white freeze-dried powder form (GenScript, Piscataway, NJ, USA) was used. RhBMP-2 solution was prepared according to the manufacturer's instructions, i.e., 0.75 mL of 20 mMol of acetic acid was gently dripped directly onto 1 mg rh-BMP2, and 0.75 mL of sterile 1xPBS was added. Then, this solution was gently dripped onto and absorbed by the PCL scaffolds. Subsequently, scaffolds were incubated in a continuously shaking machine at 250 rpm. The scaffolds were turned over at 7.5 min, and shaking continued for an additional 7.5 min. This preparation was performed under sterile conditions in the operating room, while the mandibular segmental operation was performed in each individual monkey.

Preparation of Monkey Autologous Bone Marrow Cells in Collagen Gel

After the monkeys were anesthetized, a 3-mL quantity of bone marrow was aspirated from the trochanter bone from each monkey. The aspirate was processed with red blood cell lysis and cell counting under sterile conditions.

Ultrapure Bovine Collagen Solution (Sigma-Aldrich® - C4243, St. Louis, MO, USA) was used as a carrier for bone marrow cell-seeding. Collagen gel was prepared by the pipetting of 0.8 mL of 3 mg/mL Ultrapure Bovine Collagen Solution into an Eppendorf tube. Subsequently, 0.1 mL of 10x PBS was added to the solution, followed by gentle mixing with 75 μL 0.1 M NaOH. The pH of the solution was kept in the range of pH 7 to 8. Then, a 125- μL quantity of bone marrow cells (equivalent to 5×10^6 cells) was mixed with 475 μL (pH-adjusted for appropriate gelling conditions) collagen solution. Bone marrow cells in collagen gel were incubated at 37°C, in a humidified atmosphere of 95% O_2 and 5% CO_2 , for 40-45 min.

Analysis of preliminary data from a pilot study confirmed that the collagen solution evoked no inflammatory or allergic reaction in the monkeys and proved that the viability of the marrow cells was maintained in the prepared collagen gel (data not shown).

Autologous bone marrow cells in 0.6 mL of collagen gel were transferred into the porous PCL scaffold and placed in an incubator. After 15 min, the outer surface

of the cell-seeded scaffold was coated with an additional 0.4 mL of collagen gel. The construct was re-incubated for another 30 min before placement into the mandibular defect site.

Surgical Procedure

The monkeys were fasted overnight and received 0.05 mg/kg of intravenous atropine and 10 mg/kg of Ketamine (Parnell Laboratories, Alexandria, Australia) pre-operatively. All animals were weighed prior to surgery. Induction and maintenance of anesthesia were performed with 2% isoflurane. Endotracheal intubation was performed with oral endotracheal tubes (gauge, 3.5 mm). Intravenous analgesic 2 mg/kg carprofen (Rimadly®) (Pfizer Inc, NY, USA) and antibiotics (ampicillin/cloxacillin) 6-8 mg/kg were administered. The surgical site was disinfected with 1% Cetrimide, followed by 0.05% chlorhexidine and povidone iodine, and sterile drapes were used. An incision was made intra-orally, beginning with two vertical incisions between the second bicuspid and the first molar as well as behind the second molar. A horizontal incision 2-3 mm below the attached gingiva was made to connect the two vertical incisions. The periosteum was reflected to expose the lower border of the mandible at the ostectomy sites. A tapered fissure bur was used to perform the resection, and the block was subsequently removed. A 15-mm section of the segmental defect was taken from the right side of the mandible. Bleeding from the inferior alveolar artery and vein was easily controlled with diathermy when necessary.

Three experimental groups were created: (1) PCL scaffold with CHA surface coating and soaked with rhBMP-2 (PCL-BMP, n = 8); (2) PCL scaffold with CHA surface coating and seeded with bone marrow cells (PCL-CELL, n = 8); and (3) PCL scaffold with CHA surface coating, as a control group (PCL-control, n = 8). Before installation of the scaffolds, the medullar space of the anterior and posterior bone stumps was prepared with a tapered drill (2.3 mm in diameter) to a depth of 12 mm, to conform to the dimensions of the stems of the PCL scaffolds. The stems of the anterior and posterior modules were then inserted into the prepared grooves and press-fitted, and the stability was checked. The anterior and posterior modules were then connected and stabilized with a vertical pin (Fig. 2).

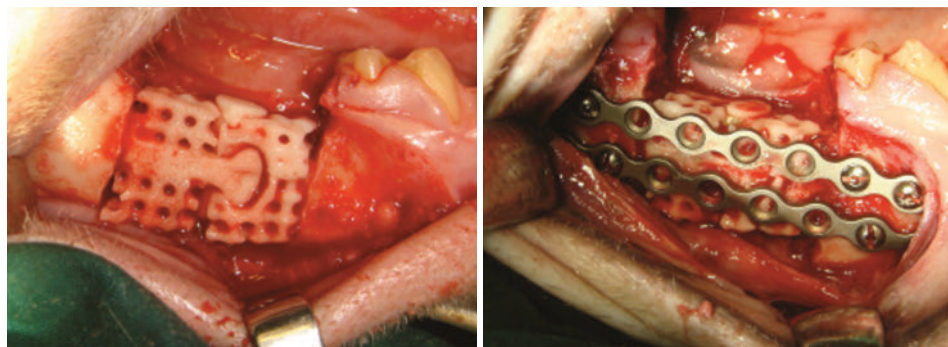


Figure 2. Clinical images of segmental mandibular reconstruction with PCL Endoprosthesis. The mandibular segment is stabilized by two titanium mini-plates and -screws.

After insertion of the endoprosthesis, occlusion was evaluated. Since intermaxillary fixation was not possible in this animal model, two Ti mini-plates with 5-mm Ti screws were fixed between mandibular stumps to immobilize the reconstruction site. The buccinator and mylohyoid muscles were dissected, mobilized, and sutured over the device by means of 4/0 Vicryl® (Ethicon Inc., Somerville, NJ, USA), followed by closure of the mucosa, thus creating a 2-layer closure. After surgery, radiographs were taken with a Siemens Polymobil Plus machine set at 40 kV for 2 ms at a distance of 70 cm. During imaging, animals were positioned with their right mandibles adjacent to the plate.

The animals were maintained in individual cages. Soft diet was provided until sacrifice. Ampicillin/cloxacillin 6-8 mg/kg IM was administered for 7 days post-surgery, and Ketorolac trometamol (Toradol) 15-30 mg/kg IM was given for 2-3 days post-surgery.

Endoprosthesis Retrieval

All animals were weighed and sacrificed at 6 months post-operatively. Mandibular specimens from condyle to condyle, with the device in situ, were harvested. Surrounding soft tissue was removed except around the reconstructed site. Radiographic examination was performed at the same settings as used for the preliminary assessment.

In half of the animals of each group, mandibles were harvested fresh to be used for mechanical testing. A 3-mL quantity of pentobarbitone was injected into the cardiac chamber to euthanize the animals. All retrieved specimens were kept frozen at -20°C until needed for analysis.

For the other half of the animals in each group, harvesting was done after perfusion fixation. A 16-gauge intravenous catheter was inserted into the left ventricle and used for circulation with 300-500 mL of Hartman's solution, followed by 750 mL of a mixture of 2.5% paraformaldehyde and 2% glutaraldehyde. The specimens were kept soaked in 10% glutaraldehyde.

Micro-CT Evaluation

The specimens containing the reconstruction device were scanned in a GE eXplore Locus SP MicroCT scanner (GE Healthcare, Thermo Scientific, Waltham, MA, USA), with a focal spot of 8 μm , pixel size of 18 μm , scanning configuration isotropic voxels of 8 x 8 x 8 μm focal spot size, and isotropic resolutions at 8 μm .

Mini-plates and screws were removed before the scanning process, to avoid scattering due to the presence of metal. The area of scan was extended to the maximum diameter of the scan view and beyond the region of interest (ROI) and the reconstruction site, which included end-points of the anterior and posterior scaffold stems. The digitized signals were then transferred to a computer for reconstruction of the micro-CT slices. Standardized calibration was used for comparison with bone, air, and water. All images were calibrated in Hounsfield units (HU) for quantitative analysis. The new bone analysis was analyzed with MicroView® 2.2 software (GE Healthcare, Waukesha, WI, USA) and Mimics® Software (Mimics 14.01 64-bit, Materialise, Leuven, Belgium).

Micro-CT slices were reconstructed perpendicular to the long axis of the mandibular reconstruction. Bone union of the mandibular stem was evaluated. Bone volume (%) at the reconstruction site was identified with Microview computer software. To investigate new bone formation inside the scaffold, we used the Stereo Lithography (STL) digital data of the scaffold to indicate the region of interest (ROI) as the boundary of the scaffold body, with Mimics® Software. The created ROI was transferred to the individual scan of the specimen in the MicroView® program. Bone volume (%), tissue mineral density (TMD) value (mg/cc), and bone mineral density (BMD) (mg/cc) were then evaluated (Fig. 3).

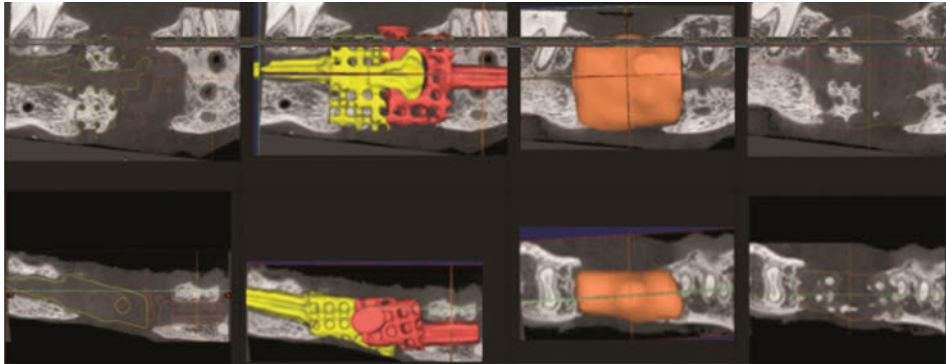


Figure 3. The region of interest (ROI) inside the scaffold was created along the boundary of the scaffold body with the Mimics x64 14.0 computer program. The quantity of newly regenerated bone inside the body of the scaffold was analyzed with MicroView™ 2.2 GE Healthcare computer software.

Mechanical Testing Examination

The specimens for mechanical testing were processed at the Biomedical Engineering Laboratory, College of Engineering, The University of Michigan, Ann Arbor, MI, USA. The three-point bending test was used to determine their stiffness in an MTS Alliance RT/30 Elite™ Controller testing machine (TestResources Inc., Shakopee, MN, USA). Each specimen was placed on the biomechanical 3-point bending test fixture, with both condyles and the mid-anterior lingual bone surface placed on a custom-made jig (Fig. 4). A force at a constant displacement rate of 25 mm/min was applied to the lower border of the mandibular body. The load-displacement data were recorded at a frequency of 15 Hz, for determination of the stiffness of the reconstructed mandibles without breaking the specimens.

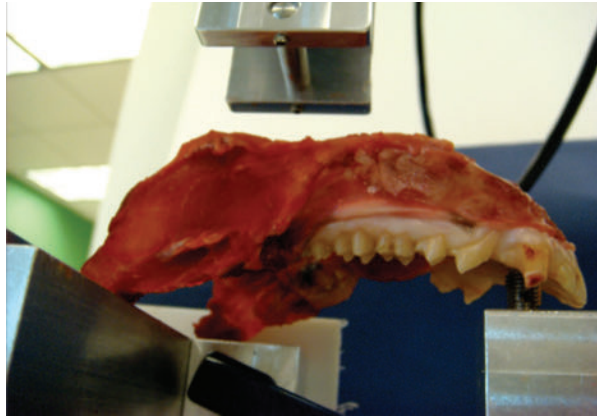


Figure 4. Each of the mandibles was placed on the 3-point bending jig for biomechanical testing with the Alliance RT/30 Elite™ Controller. The jig model was designed by the Department of Biomedical Engineering, University of Michigan, Ann Arbor, USA. The mechanical testing system was exposed to compression loads that simulated masticatory loads on each side of the mandible (reconstructed and non-reconstructed sides). Vertical linear displacement was applied by the Alliance RT/30 Elite™ Controller machine.

A pilot study was conducted to determine the maximum load that could be applied without mandibular fracture. The data were acquired from 2 dry intact macaque mandibles. The first dry mandible was placed on the biomechanical 3-point bending testing fixture as above. The vertical linear displacement was applied by vertical load on one side of the mandibular body. Once the pre-load was reached, data were acquired at a rate of 15 Hz, while a load was applied at a displacement rate of 0.25 mm/min until the failure load resulted in mandibular fracture at a loading force of 889.6 N. The second dry mandibular specimen was tested similarly, except that the maximum load was reduced to 111.21 N, or 12.5% of the failure load, to avoid mandibular fracture. The stiffness of the mandibular specimen was found to be 420 N/mm and 643.7 N/mm on the contralateral side without fracture.

Before mechanical analysis, the harvested specimens were thawed to room temperature from -20°C for 2 hrs. All specimens were maintained in moist conditions until the test was completed. Before mechanical testing, mini-plates and screws were removed, including most of the soft tissue around the mandible. Bilateral mandibular coronoid processes and canine cusps were trimmed to prevent interference with the fixation jig during mechanical testing. Into the anterior lingual bone of each mandible, a 3-mm hemisphere was drilled by means of a surgical round bur to prevent displacement of the specimen during force application.

Mechanical testing was done for both sides of the mandible at a displacement rate of 0.25 mm/min until 111.201 N was reached. The maximum applied moment (MAM) or maximum moment at failure (MMF) point due to the conclusion of bending force application was stopped before the breaking failure point of each specimen. Force and displacement as well as elastic stiffness (N/mm) were recorded. Unstable mandibular specimens were excluded from mechanical testing. After mechanical analysis, specimens were immersed in 10% formaldehyde for subsequent histological preparation and analysis.

Histological Analysis

All histological processing and specimen analyses were performed at the Department of Biomaterials of the Radboud University Nijmegen Medical Centre, The Netherlands.

The specimens were reduced in size, dehydrated in a graded series of ethanol, embedded in methyl methacrylate resin, and polymerized. The tissue blocks were mounted in a modified inner circular saw microtome (Leica® RM 2165, Wetzlar, Germany), and 10- μ m-thick sections were prepared. Serial bucco-lingual cross-sections were stained with methylene blue and basic fuchsin for histology and histomorphometric analysis. At least 7 bucco-lingual histological cross-sections were prepared from the reconstructed mandible from each specimen, i.e., 1 at the midline of the device's body, 3 at the junction between the device body and the stem, and 3 at the midpoint of the scaffold's stem (Fig. 5).

Light microscopy (Leica®, Rijswijk, The Netherlands) was used for histological evaluation, which included a general description of the tissue surrounding the implant's body, the junction of the body and the scaffold's stem, and the stem areas.

Histomorphometric analysis was performed by one observer (CN). A modified hard-tissue histologic grading scale³¹ (Table I) was used to quantify the histological findings. The score of each sample was calculated. Total bone contact (TBC) (%) and bone volume (BV) (%) were measured for at least 3 sections of the stem, junction, and mid-scaffold.

Table I. Hard-tissue Histologic Grading Scale
(adapted and modified from Jansen *et al.*, 1994)

Parameter	Score
Bone Formation	2 : Completely surrounded with bone 1 : Partially surrounded with bone 0 : No bone (fibrous formation)
Bone-Device Interface	2 : Completely interfaced with bone 1 : Partially interfaced with bone 0 : No bone (fibrous formation)
Scaffold Degradation	3 : Completely disappeared - almost complete degradation or complete fragmentation 2 : Marked degradation - marked cracks in implant and/or some fragments toward edges and outer surface 1 : Limited degradation - some minor dissolution on edges, minor cracks in implant, and/or small fragments present 0 : No degradation - Completely intact polymer

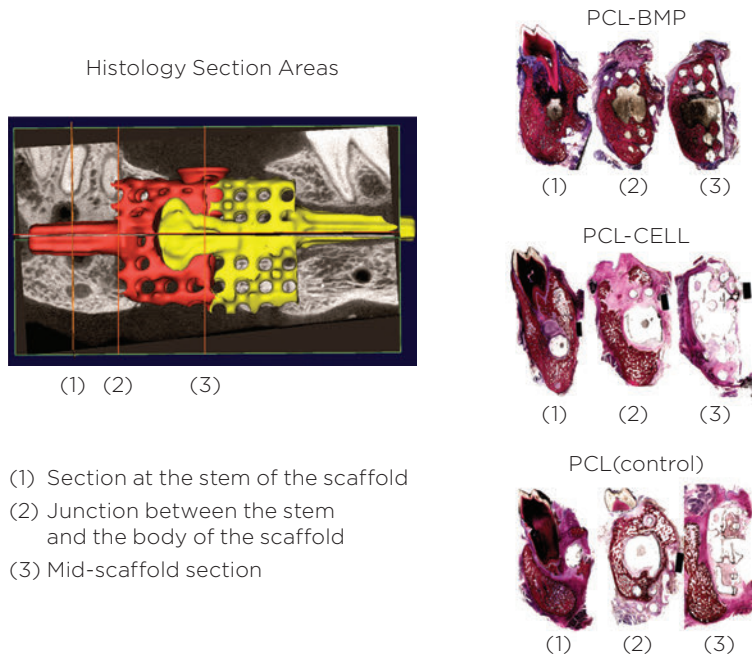


Figure 5. Histology slide sections (magnification 1x) from 3 areas of the scaffold: (1) the stem of the scaffold, (2) the junction between the stem and the body of the scaffold, (3) and a mid-scaffold section. The sections showed better bone formation inside the scaffold structure of the PCL-BMP group, while mostly fibrous tissue was found in the PCL-CELL and PCL-control groups. The stem of the scaffold in the PCL-BMP group was surrounded with bone, while fibrous connective tissue was again found in the other two groups.

Total bone contact (TBC) (%) at the stem was quantified by microscopy at 5x and 10x magnification (Zeiss® computer program). TBC (%) was calculated with the Image-Pro® 5.0 system (Media Cybernetics, Silver Spring, MD, USA). TBC (%) was calculated according to the formula:

$$\text{TBC (\%)} = \frac{\text{Total length of bone interface}}{\text{Total length of stem surface}} \times 100$$

The mean bone volumes (%) were calculated 2 mm from the stem surface (Fig. 6). Bone volumes were calculated with the QWin computer program (Q-win;Leica®, Wetzlar, Germany) using the average value of 3 parallel slices and according to the formula:

$$\text{Bone Volume (\%)} = \frac{\text{Total Bone Volume (- area of tooth or plate/screw)}}{\text{Calculated area 2 mm from the stem surface}} \times 100$$

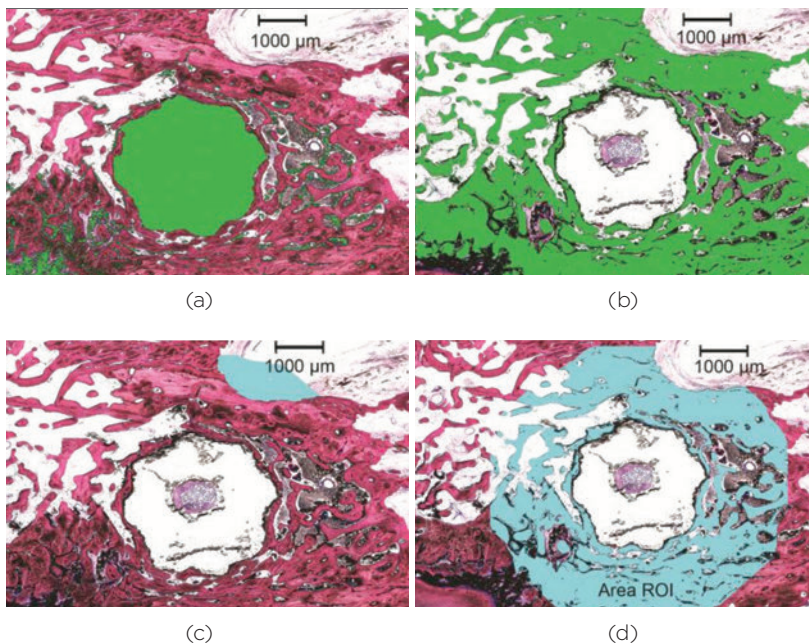


Figure 6. The Qwin computer program was used to calculate the percent bone volume from the area 2 mm around the stem implant surface. The images show the method for identification of the region of interest (ROI). The computer program identified: (a) the stem area. (b) the bone around the stem, and (c) removal of other defects, i.e., screw or root tip. The selected area (ROI) around the scaffold's stem is used to calculate the percent bone volume, as shown in (d).

Statistical Analysis

Data from the mechanical tests were described by stiffness values.

Data on bone regeneration from the micro-CT study and data on histological measurements were statistically analyzed with SAS 9.2 statistical software (SAS Institute Inc., Cary, NC, USA). Measurements were evaluated by Analysis of Variance (ANOVA) with a pair-wise comparison post-test to identify the groups that differed from each other. This was done without correction for Type I error rate across the pair-wise tests. A P -value < 0.10 was considered statistically significant.

Results

Gross View and Clinical Findings

Although all 24 animals survived the experimental period and maintained their body weight well, at retrieval only 14 specimens were found to be useful for further assessment (PCL (control) ($n = 3$), PCL-BMP ($n = 6$) and PCL-CELL ($n = 5$)). The rest of the animals had to be excluded due to loosen plates and screw, clinical mobility and wound infection at the reconstruction sites (Fig. 7).

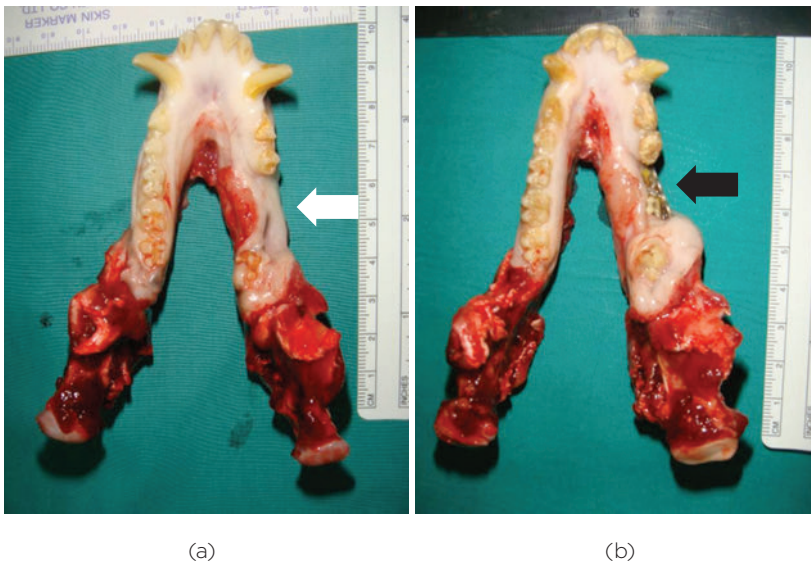


Figure 7. Images of reconstructed mandibular specimens at 6 months. Several of the specimens successfully maintained their shape, mandibular contour, and an intact oral mucosa (white arrow) (a), while others presented with wound dehiscence. The scaffold and miniplates are exposed through intraoral wounds (black arrow) (b).

Serial radiographic examinations and gross examination showed incomplete union between bone segments in all groups at 6 months after surgery. Radiographic bone formation was found to be higher in the PCL-BMP than in the PCL-CELL and PCL (control) groups, and regenerated bone was also found outside the scaffold, especially in the area adjacent to the lingual periosteum. The fixation plates and screws appeared to be loose in some specimens (Fig. 8).

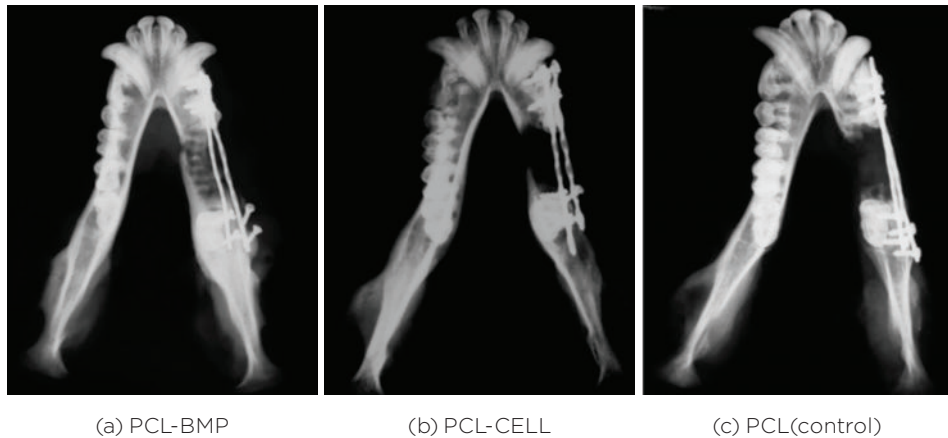


Figure 8. Radiographic images of the reconstructed monkey mandibles: (a) PCL-BMP, (b) PCL-CELL, and (c) PCL-control. The mandibles maintained both shape and dimension. The new bone regeneration was found to be nearly complete in the PCL-BMP group. Incomplete bone union was observed in the other two groups. Loosening of plates and screws was found in several specimens.

Micro-CT Analysis

Micro-CT revealed that bone regeneration in the various PCL scaffold groups never resulted in complete repair of the continuity defects at 6 months. Deformation of the scaffold, i.e., bending or fracture between the stem and the body of the scaffold, was found in two specimens (PCL-control and PCL-CELL). The lingual side of the mandibular defect showed homogeneous bone regeneration.

Calculated Mean Bone Volume (mm^3) at the reconstruction side (area between mandibular segments) was found, for PCL-control, PCL-CELL, and PCL-BMP, to be 210.07 ± 112.37 , 566.66 ± 371.30 , and $481.98 \pm 281.60 \text{ mm}^3$, respectively. However, bone formation presented mainly at the lingual side of the defect, and some regenerated bone was separated from the scaffold. Further evaluation of new bone formation inside the scaffold structures revealed a high level of bone formation in the PCL-BMP group compared with the other groups (Fig. 10). Mean Bone

Volume inside the scaffold for PCL-control, PCL-CELL, and PCL-BMP was 27.98 ± 34.64 , 9.21 ± 7.42 , and 153.45 ± 171.42 mm³, respectively.

The mean Tissue Mineral Content (TMC) for PCL-control, PCL-CELL, and PCL-BMP was 14.15 ± 17.34 , 4.87 ± 4.08 , and 82.96 ± 91.33 mg, respectively, and the mean Tissue Mineral Density (TMD) for PCL-control, PCL-CELL, and PCL-BMP was 521.48 ± 25.59 , 491.62 ± 60.73 , and 547.23 ± 40.13 mg HA/cc, respectively. Nevertheless, statistical analysis demonstrated that the observed differences in bone volume (outside and inside), TMC, and TMD between and among the various groups were not significant ($p > 0.10$) (Figs. 9, 10).

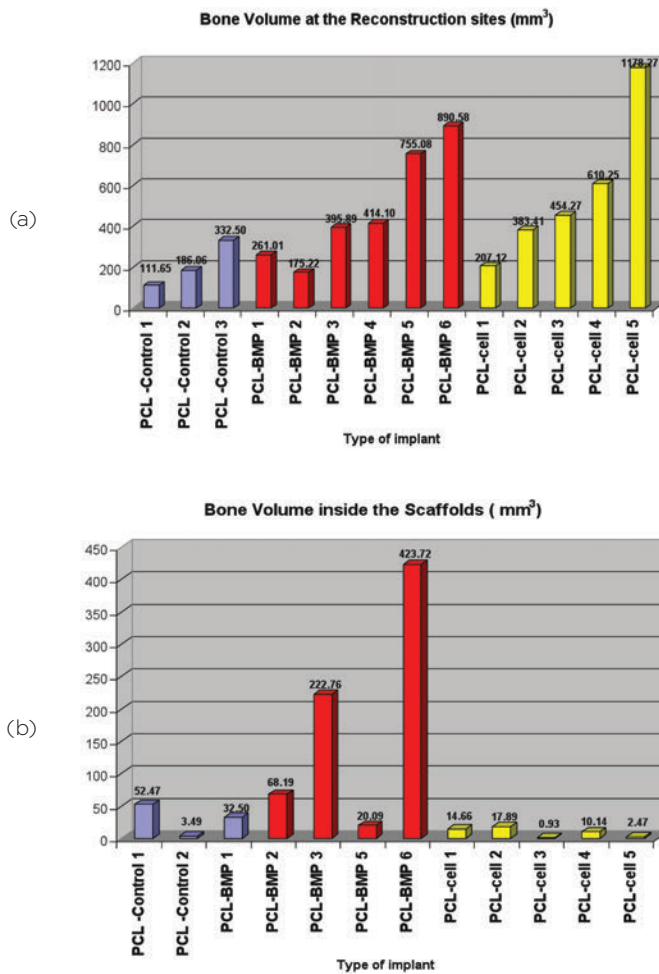


Figure 9. Micro-CT scan analysis results. a) Bone volume at the reconstruction site of each specimen. b) Bone volume formation inside the PCL scaffold in each specimen.

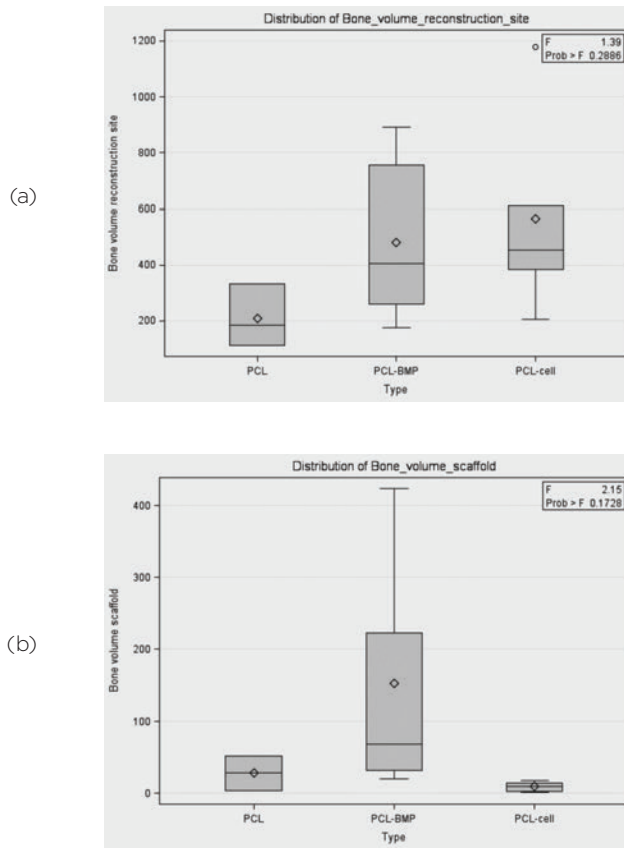


Figure 10. Micro-CT scan analysis. a) Mean bone volume at the reconstruction site of each specimen. b) Mean bone volume inside the PCL scaffold. There was no significant difference in mean bone volume at the reconstruction site and inside the scaffold among the 3 groups ($P > 0.010$).

Mechanical Testing

Successful mechanical testing could be undertaken only on 4 specimens, i.e., PCL-BMP ($n = 2$) and PCL-CELL ($n = 2$), while the other 4 specimens presented with poor stability at the reconstruction site, which did not allow for mechanical assessment. The stiffness values on the experimental side of the mandible were 7.1 and 24.4 N/mm in the PCL-CELL group and 193 and 61.9 N/mm in the PCL-BMP group. The mean stiffness values on the contralateral side of the mandible were 129.4 and 198.2 N/mm in the PCL-CELL group and 820.9 N/mm in the PCL-BMP group. The peak load, reported as 40.41 N, was found in one of the two samples from the PCL-CELL group and also in one sample of the PCL-BMP group, which was found to be at 72.09 N (Fig. 11).

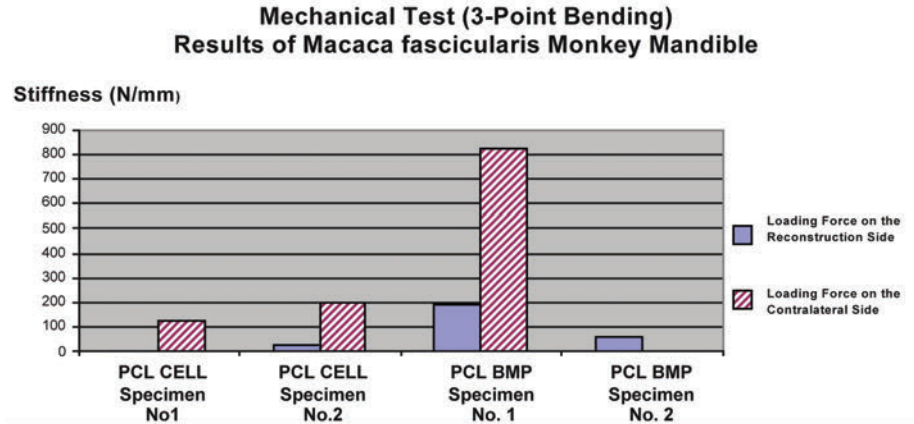


Figure 11. Stiffness values of 3-point bending in monkey mandibles. Stiffness value in the PCL-CELL is 7.1 and 24.4 N/mm while loading force was applied to the experimental side and 129.4 and 198.2 N/mm with force applied to the contralateral side. Stiffness value in PCL-BMP is 193 and 61.9 N/mm while loading force was applied to the experimental side and 820.9 N/mm with force applied at the contralateral side. Mechanical testing could not be performed on any of the PCL-control specimens, due to weakness at the reconstruction site.

Analysis by Light Microscopy

Successful histological analysis was performed on 14 specimens, i.e., PCL-BMP ($n = 6$), PCL-CELL ($n = 5$), and PCL-control ($n = 3$), while the rest of the specimens were excluded due to infection. The histological sections showed more enhanced bone regeneration in the PCL-BMP than in the PCL-CELL and PCL-control groups (Fig. 12). The PCL scaffolds showed minimal signs of degradation in all groups. In detail, the PCL-BMP group showed normal-appearing mature trabecular bone both outside and inside of the scaffold's porosity in 2 of the 6 specimens. The majority of the cells detected were osteocytes and osteoblasts. The porosity of the PCL-CELL and the control groups was mainly filled with soft tissue, with an abundant presence of fibroblasts and inflammatory cells. Islands of bone were found related to the periosteum, especially at the lingual side, outside the scaffold. At the junction of the body and scaffold stem, bone formation starting from the mandibular stump and progressing into the scaffold porosity and bone was found to be present at the lower border and lingual side of the mandible in 3 of the 6 specimens from the PCL-BMP group, 2 of 5 specimens from the PCL-CELL group, and 1 of 3 specimens from the PCL-control group. Again, the amount of bone formation in this region appeared to be higher for the PCL-BMP group.

Around the stems of the scaffolds, a thick connective tissue layer was present between the stem surface and native bone in most specimens. However, direct bone contact at the interface was found in 3 specimens of the PCL-BMP group and in 1 of the PCL-control groups.

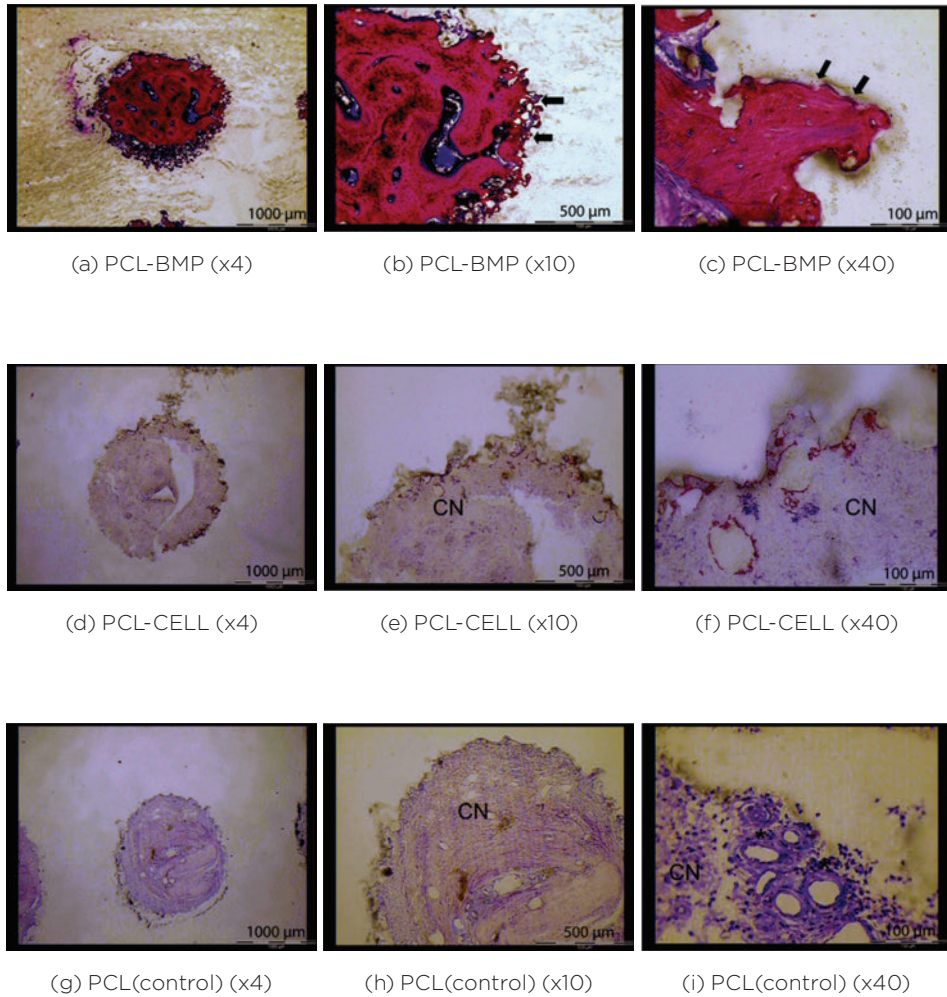


Figure 12. Histology slide sections of scaffold porosity at the mid-scaffold region: PCL-BMP (a) to (c), PCL-CELL (d) to (f), and PCL-control (g) to (i). Bone formation was found in PCL-BMP scaffold pores, while connective tissue was observed in the other two groups. Good bone-scaffold contact with areas of scaffold degradation was seen along the edge in the PCL-BMP group. CN = connective tissue, *inflammatory cells, and arrows = bone-scaffold contact areas.

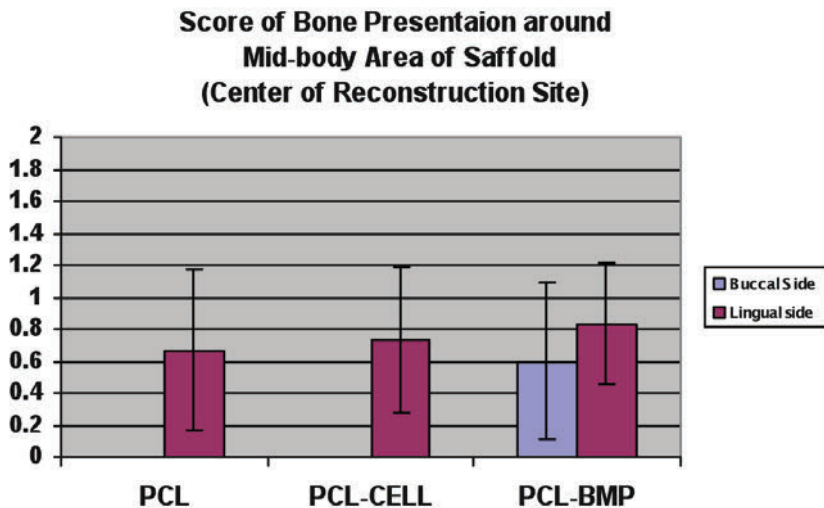
Histomorphologic Analysis

Bone formation around the device and bone-device interface was analyzed in 3 areas (mid-scaffold, junction between the scaffold body and stem, and mid-posterior stem), and the grading scale used is depicted in Figs. 13 and 14.

The mean total bone presentation grading score for the 'area around the devices' was 0.69 ± 0.01 for the PCL-control group, 0.63 ± 0.01 for the PCL-CELL group, and 1.21 ± 0.29 for the PCL-BMP group. The difference in total bone presentation score between the PCL-BMP and PCL-control groups, and also between the PCL-BMP and PCL-CELL groups, was found to be statistically significant ($p < 0.10$). There was no statistically significant difference between the PCL-control and PCL-CELL groups.

The mean total grading scores for the 'bone-device interface' were 0.19 ± 0.29 for the PCL-control group, 0.12 ± 0.15 for the PCL-CELL group, and 0.55 ± 0.46 for the PCL-BMP group. Statistical testing revealed no significant differences in the mean total grading scores between and among groups.

The percentages of bone-device interface calculated around the stem of the implant to the native bone were $2.26\% \pm 5.30$ for the PCL-control group and $2.96\% \pm 5.01$ for the PCL-BMP group, and there was no bone-device interface (0%) in the PCL-CELL group. There were no statistically significant differences between the PCL-control and PCL-BMP groups.



(a)

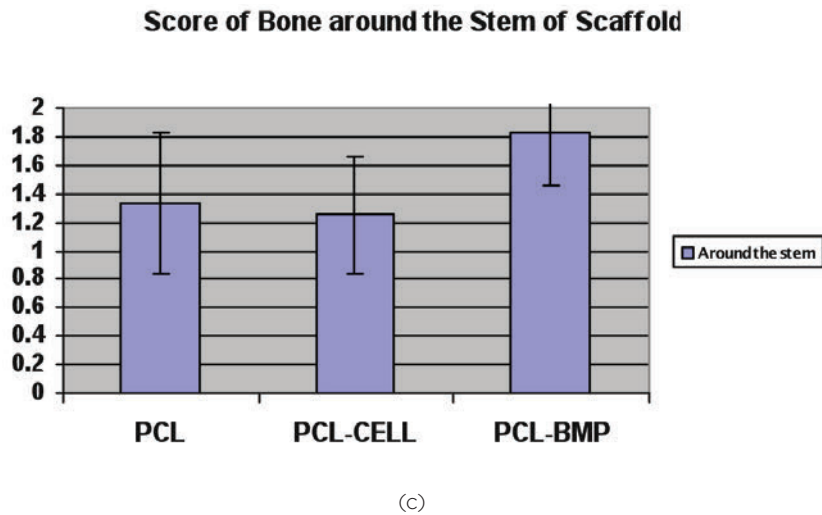
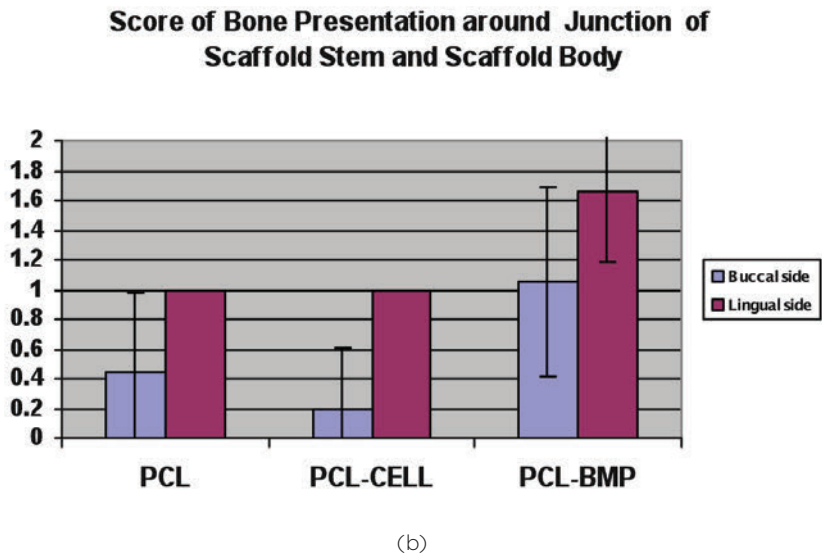


Figure 13. Bone present around the scaffolds: a) mid-body, b) junction, and c) scaffold stem. The score scale: 0, no bone (fibrous formation); 1, partially surrounded with bone; and 2, completely surrounded with bone.

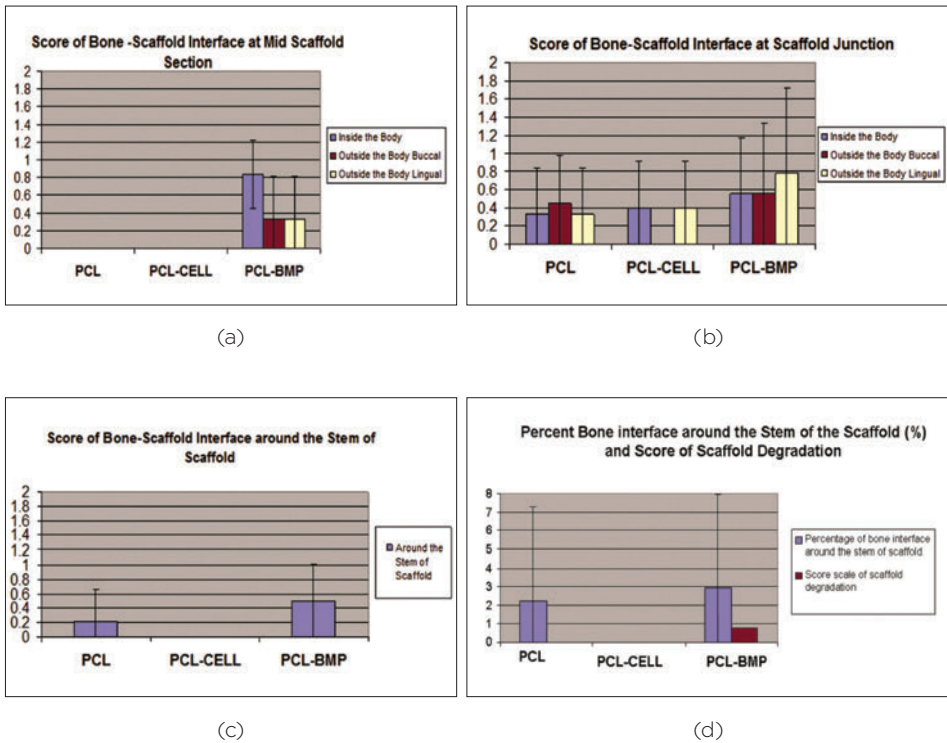


Figure 14. Scoring of bone-scaffold interface at 3 areas: a) mid-body, b) junction, and c) scaffold stem. 0, No bone (fibrous formation); 1, partial interface with bone; and 2, complete interface with bone. Percentage of bone interface around the stem of the scaffold and scoring of scaffold degradation are shown in (d). Score scale for scaffold degradation is described as: 0, no degradation; 1, limited degradation; 2, severe degradation; and 3, bone has completely disappeared.

The mean scores for scaffold degradation were 0 for the PCL-control group, 0 for the PCL-CELL group, and 0.78 ± 0.43 for PCL-BMP groups (Fig. 14d).

The results of the bone contact and bone volume percentages, as determined with image analysis software, are depicted in Fig. 15. The differences in mean percent bone volume (%), calculated in the area of 2 mm around the stem implant surface, were $27.67\% \pm 0.19$ for the PCL-control group, $32.65\% \pm 15.58$ for the PCL-CELL group, and $56.33\% \pm 6.98$ for the PCL-BMP groups.

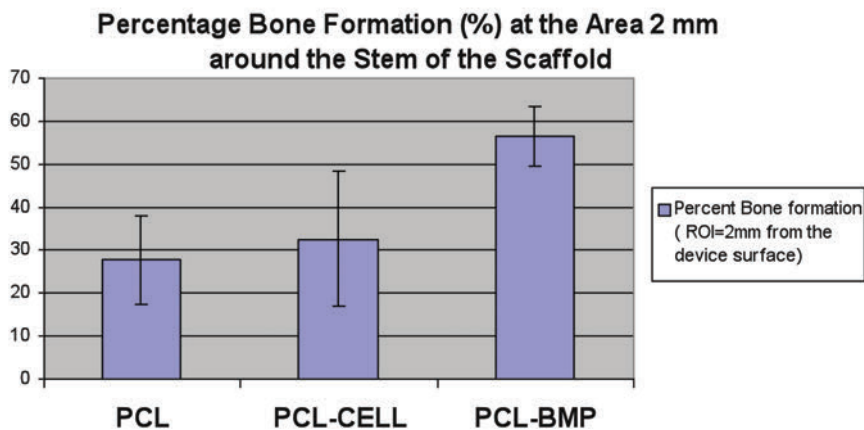


Figure 15. Percentage bone volume (%) calculated from the ROI (area 2 mm around the scaffold stems).

The mean percent bone volume between the PCL-BMP and PCL-CELL groups and between the PCL-BMP and PCL-control groups was found to be statistically significant ($p < 0.10$) among the groups. There was no statistically significant difference between the PCL-control and PCL-CELL groups.

Discussion

This study is the first attempt to reconstruct a mandibular body segmental defect using the endoprosthesis-designed PCL scaffold combined with either rhBMP-2 or autologous bone marrow cells in a non-human primate model. The continuity defect of segmental resection in the study was similar to those resulting from ablative surgery, e.g., trauma, tumors, or osteomyelitis in the oral and maxillofacial region. We proposed to compare the degrees of bone regeneration that occurred from the PCL scaffold reconstruction resulting from the addition of either rhBMP-2 or autologous bone marrow cells.

Macaca fascicularis monkeys were selected in this study due to their anatomic and biological mandibular similarity to humans.^{3, 32} The six-month follow-up period was considered suitable based on results from previous studies on the similar healing of a defect site in the same model evaluating bone bridge formation in a mandibular continuity defect by a tissue-engineering technique,^{16, 33-36} including reconstruction with a titanium alloy modular endoprosthesis.⁵ Although the non-primate human model is closed to clinical study, the immobilized jaw movement

by the inter-maxillary fixation (IMF) cannot be performed, unlike in humans. Therefore, the identification of an alternative method for early stabilization of the reconstruction site was crucial. Although the addition of internal fixation with 2 mini-plates and screws was used to maintain the mandibular integrity of bone segments in the study, unfortunately, the results demonstrated insufficient load-bearing capacity in most of the animals, and, subsequently, infection was found in many specimens.

The immediate loading force and the intra-oral surgical approach appeared to be related to wound dehiscence and disrupted the achievement of bone union. Wound dehiscence was experienced by the animals as an 'uncomfortable feeling' and resulted in additional disturbance of the wounds by the monkeys with their fingers. Wound dehiscence as well as dislodgement of the mini-plates and screws led to a limited number of appropriate specimens for further evaluation. Therefore, the data should be interpreted with caution.

There was incomplete bone union in all study groups; therefore, we rejected the hypothesis that an osteoinductive scaffold loaded with rhBMP-2 or bone marrow cells could achieve bone union and overlying soft-tissue healing with sufficient load-bearing capacity within 6 months. However, the findings showed that the amount of bone in-growth was higher in the PCL-BMP-2 group compared with that in the PCL-CELL and PCL-control groups. The micro-CT imaging in one specimen from the PCL-BMP group showed a nearly complete bone union with bone in-growth. The mechanical test showed that the mandible reconstructed with PCL-BMP had a higher load-bearing capacity compared with that of the other groups. Nonetheless, with the limitation that the study was discontinued at 6 months, it might be possible that bone formation and bone union in the PCL-BMP group may or may not continue if a follow-up period was set at more than 6 months. However, based on the current findings, the PLC-BMP-2 reconstruction has potential for bone regeneration in mandibular continuity defects.

Basic bone bioengineering can be accomplished relative to many factors, including bone scaffold, growth factors, biologic cells, and surrounding vascular blood supply, especially in large reconstruction sites.^{37, 38} The ideal biomaterial properties for bone scaffolds were identified as biocompatibility, a capacity for facilitating revascularization, osteoinductive and osteoconductive properties, and a structure providing a framework for new bone development while allowing for the incorporation of osteogenic factors.³⁸ Furthermore, the material should be easily shaped into complex components, as well as being malleable, sterilizable, storable, and affordable. The material stiffness should offer an initial primary stability for

the reconstruction site with subsequent gradual degradation corresponding with newly deposited bone in-growth, to maintain the proper load-bearing capacity. The candidate scaffold materials which closely fulfill such requirements are bioresorbable aliphatic polyesters, such as polyglycolide, polylactide, PCL, and their copolymers.

PCL is the FDA-approved material used in multiple medical device formulations.¹² It already has a significant history of regulatory approval, with minimal inflammatory and immunological responses,^{39, 40} and has been used in clinical applications highly biocompatible with osteoblasts. The thermoplastic quality of PCL allows it to be processed in 3 dimensions with the desired geometry, and for controlled porosity with interconnectivity by modern computer-based solid free-form fabrication technology. In the current study, the scaffold was designed to follow the anatomy of a monkey mandible based on the computed scan (CT) data. It was comprised of a body segment with two modular endoprosthesis stems inserted into the prepared cancellous cavity of a native mandible. The PCL scaffold was intended to maintain the stability of the mandible and to support the anatomical regeneration of the bone defect.

Among growth factors for the enhancement of bone regeneration, BMPs have been reported to be successful in bone reconstruction. The selected carriers reported in the literature were collagen sponges^{16, 35, 41, 42}, poly-D, L-lactic-co-glycolic acid-coated gelatin sponges (PGS)⁴³, poly D,L-lactic coglycolic acid (PLGA)-coated gelatin sponges (PGS)⁴⁴, polyglycolic co-lactic acid (PGLA)⁴⁵, and autologous bone graft of freeze-dried bone.⁴⁶ Among these materials, the collagen type-I sponge was found to be the most frequently used in preclinical and clinical studies in segmental mandibular reconstruction with promising results. However, the collagen sponge lacks sufficient structural integrity to maintain the defect space compared with a bone graft and also lacks loading capacity.¹⁸ Therefore, there is great interest in the search for other bone scaffold carriers better suited to bone defect repair.¹⁸ PCL is a candidate material. Although it has non-osteoinductive properties, an engineered CHA surface coating on PCL has been proven to allow protein molecules such as rhBMP-2 to attach and be released in a controlled manner,^{12, 13} as was used in this study.

The porosity of the scaffold functions as a repository for housing bone marrow mesenchymal cells to be transported to the reconstruction site. The autologous bone marrow cells utilized in this study were aspirated from the autologous trochanter bone, since we noted that the monkey's iliac bone size was small. The autologous bone marrow cells were processed and seeded into the scaffolds for reconstruction

without the *in vitro* incubation process, so that the bone reconstruction process could be completed within the same operation. This approach was found to compromise the function and morphogenic ability of the bone marrow cells possibly related to poor cell viability after implantation. The bone regeneration results in the PCL-CELL group were complicated by wound infection without bone in-growth. The lack of bone formation in the center of the scaffold possibly resulted from insufficient blood supply to the inner side of the scaffold structure, required to maintain cell survival. The results therefore did not differ from those of the control group. The conclusions resulting from the use of a PCL scaffold combined with autologous bone marrow cells yielded insufficient supporting data. The methodology on cellular approaches to bone regeneration requires further study.

The acknowledged slow degradation phenomenon of PCL, which depends on random hydrolytic chain scission of the ester linkages, may vary from months to years.⁴⁷ Polymer degradation can be characterized as a decrease in the rate of chain scission and the onset of implant weight loss, fragmentation, and intracellular degradation.⁴⁸ This slow degradation could even hamper bone in-growth. The PCL scaffold degradation in the current study was limited, and the remaining structure was still present at the end of the study. However, the actual amount of PCL degradation, e.g., molecular weight loss, was not evaluated in this study.

Further studies will be needed to determine the optimal methodology and parameters prior to clinical use. In future studies, additional steps can be taken to increase the stability of the endoprosthesis. A surgical approach to mandibular reconstruction should preferably be performed extra-orally to reduce wound dehiscence and infection rates. PCL can be the material of choice; however, after insertion into the mandible, the modular components of the scaffold can be heat-welded together to increase the rigidity of the prosthesis. Importantly, sufficient rigid plate(s) fixation should be used to improve the primary stability of the reconstructed site, especially in the initial stage of reconstruction. The protocol for bone marrow cell preparation and seeding requires revision if promising results are to be achieved.

Conclusion

In conclusion, the results of our study did not confirm the original hypothesis. Based on the data obtained, no satisfactory bone formation occurred between the mandibular segments at 6 months after surgery in any of the three groups. There was a high rate of infection and dislodgement of the fixation plates and the PCL endoprosthesis scaffold. Nevertheless, the BMP-2-loaded PCL scaffolds were found to perform better in terms of bone formation and mechanical testing than empty PCL scaffolds and scaffolds loaded with autogenous BMSCs. This suggests that this might be a feasible approach for further study in reconstructing segmental mandibular defects.

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Disclosure Statement

Prof. Stephen E. Feinberg is a founding member and Dr. Leenaporn Jongpaiboonkit is an employee of the biotechnology company, Tissue Regeneration Systems, which manufactures coated PCL scaffolds that are similar to the scaffolds used in this study. Other authors declare no conflict of interest.

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THE USE OF A POLYCAPROLACTONE- TRICALCIUM PHOSPHATE (PCL-TCP) SCAFFOLD FOR BONE REGENERATION OF TOOTH SOCKET FACIAL WALL DEFECTS AND SIMULTANEOUS IMMEDIATE DENTAL IMPLANT PLACEMENT IN *MACACA FASCICULARIS*

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Introduction

Dental implants placed in fresh sockets after tooth extraction are called ‘immediate’ implants.¹ Survival rate of implants placed using this technique has been shown, in a systematic review, to be comparable with immediate-delayed and delayed implants.² The advantages of immediate implants include the reduction in the number of surgical interventions and total treatment time.^{3, 4} The incongruity, however, between the shape and size of the dental implant and that of the socket wall may lead to gaps or peri-implant defects. It has been found that peri-implant defects of 2mm or less healed by spontaneous bone regeneration and defect resolution. In the presence of peri-implant defects larger than 2mm, concomitant augmentation procedures were required to achieve an optimal outcome.⁵

Localised pathologic processes may lead to damage of one or more walls of the extraction socket, with the formation of dehiscence defects.⁶ Examples include traumatic injuries resulting in dentoalveolar fracture, periapical granulomas or cysts causing resorption of the alveolar bone and periodontal disease. The socket wall may also be damaged in the process of exodontia. Clot stabilisation and bone formation after extraction of the involved teeth may be adversely affected by the lack of intact bony walls. Bone regeneration and aesthetic outcomes may thus be compromised when immediate implants are placed at these sites. Various types of barrier membranes and/or bone grafts have been commonly used for augmentation of these defects at the time of immediate implant placement. Defect reduction using these methods ranges from 48% to 77.4%.^{6,7} The use of autogenous bone is complicated by donor site morbidity and bone resorption over time. Allogeneic and xenographic bone have been used, therefore, as alternative graft materials to autogenous bone. A study reported that tooth sockets grafted with bovine (Bio-Oss) material were comprised of connective tissue and only 40% of the circumference of the Bio-Oss particles were in contact with woven bone.⁸ Another study using demineralised freeze-dried bone allograft (DFDBA) in tooth extraction sockets showed non-viable particles of DFDBA with no evidence of bone formation on the surfaces of the implanted particles.⁹

A randomized controlled trial using various augmentation techniques at the time of immediate implant placement showed that a greater horizontal resorption of the facial bone occurred in the presence of a dehiscence defect as compared to intact sites.¹⁰ Another study reported a high incidence of recession of the labial mucosa following immediate implant placement at sites with a facial bone defect, despite augmentation using autogenous bone or Bio-Oss and collagen membrane.¹¹

A systematic review also found strong evidence that regenerative outcomes using conventional augmentation materials and techniques were less successful when dental implants were placed in sockets with dehiscence defects as compared to those with intact bone walls.¹²

Polycaprolactone (PCL) is regarded as a non-toxic and tissue-compatible material¹³ and has been used in many medical devices for the last 30 years. It was used in Capronor®, a one-year implantable subdermal contraceptive device¹⁴ and in bioresorbable Monocryl® monofilament sutures¹⁵. Both are FDA-approved clinical products. More recently it has been approved as a bone filler for craniofacial applications (510K FDA K051093) (http://www.accessdata.fda.gov/cdrh_docs/pdf5/K051093.pdf). The PCL scaffold is intended for use in the repair of neurosurgical burr holes, craniotomy cuts and other cranial defects and in the augmentation or restoration of bony contour in the craniofacial skeleton¹⁶. An interdisciplinary group at the National University of Singapore, in collaboration with Temasek Polytechnic, evaluated and patented the parameters used to process PCL and PCL composites by Fused Deposition Modelling (FDM).¹⁷ The unique feature of these FDM scaffolds lies in the 3-angle layering (0°/60°/120°) that results in a fully interconnected matrix architecture that provides maximum anchorage for cell attachment.¹⁷⁻¹⁹ The scaffold has mechanical properties closely similar to bone, exhibits slow degradation kinetics, enhances blood clot entrapment and vascular ingrowth and is osteoconductive. The second-generation scaffolds produced by FDM are based on composites. Bioactive composite 3-dimensional (3D) scaffolds comprising of a biodegradable polymeric phase and a bioactive phase that can bond spontaneously to and integrate with bone are recent innovations in the field of regenerative medicine. It was hypothesized that PCL-TCP scaffolds possess cell and protein binding sites due to the nucleation of the tricalcium phosphate on its surface.^{20, 21} The PCL-TCP scaffold was shown to have improved mechanical and biochemical properties as well as more favourable degradation and resorption kinetics than the first generation scaffolds.²²⁻²⁴ The use of PCL-TCP scaffolds for the treatment of bone defects has been tested in several animal studies.^{25, 26} It was postulated that these scaffolds would be suitable for reconstruction of tooth socket dehiscence defects during immediate dental implant placement because they: 1) eliminate the need for an autogenous donor site; 2) are available in unlimited quantity and consistent quality; 3) have a highly porous and honeycomb-like architecture that facilitates the infiltration of new osteoid; 4) do not evoke an undesirable prolonged inflammatory response; and 5) can withstand local mechanical stresses.²⁵

The hypothesis for this study was that the insertion of a 3D bioresorbable PCL-TCP scaffold into tooth extraction sockets with a facial wall defect and simultaneous immediate dental implant placement would result in favourable bone regeneration of the defect, allowing optimal bone-to-implant contact and dental implant stability. The study compared in a monkey model (1) peri-implant bone healing and regeneration, (2) bone-to-implant contact and (3) dental implant stability, following immediate implant placement into tooth sockets with surgically created facial wall defects in the following treatment groups:

- (a) use of a 3D PCL-TCP scaffold as space filler (test group)
- (b) use of particulate autogenous bone as a graft (control group)

Material and Methods

Scaffold Fabrication

The PCL-20% TCP scaffolds were purchased from Osteopore International (Singapore). The TCP powder particle size was determined by Coulter Laser Diffraction LS100Q to be less than $63\text{ }\mu\text{m}^{27}$. A composition of 20% TCP by weight was selected as it was considered to be adequate to reinforce the PCL while minimizing the risk of problems arising from the rheological properties during the micro-extrusion process used in the fabrication of the scaffolds. The scaffolds were fabricated by the latest FDM techniques (FDM 3000; Stratasys, Eden Prairie, MN), in a class 10K clean room environment. Each scaffold had a lay-down pattern of $0^{\circ}/60^{\circ}/120^{\circ}$, porosity of 70%, and measured 8mm x 8mm x 6mm. The PCL-TCP scaffolds had a typical honeycomb structure with interconnected equilateral triangles of regular porous morphology. They were individually packed and sterilized using gamma irradiation. (Table 1)

Table 1. Properties of the PCL-20%TCP scaffold

PCL-TCP Properties (Osteopore International Pte Ltd)	
PCL grade	FDA approved medical grade
PCL mol weight	143850 g/mol
PCL weight %	80
TCP weight %	20
TCP particle size	60-40 Qm
Manufacturing method	Computer controlled micro extrusion
Manufacturing environment	Clean Rm Class 10K
Sterilisation method	Gamma irradiation
PCL-TCP lay down pattern	0/60/120
PCL-TCP rod size	0.15 mm
PCL-TCP porosity	70%
PCL-TCP pore size	500 Qm
PCL-TCP stiffness	6.8 MPa
PCL-TCP yield stress	1 MPa

Dental Implant

Biomet 3i® (Biomet, Florida, USA) Nanotite tapered Prevail (expanded platform) dental implants (NIIOS3413; implant body diameter 3.25 mm, occlusal diameter 4.1 mm, apical diameter 2.4 mm and surface height 12.52 mm) were used. The titanium surfaces were dual acid-etched and with Discrete Crystalline Deposition™ (DCD™) of nano-scale calcium phosphate.

Surgery

18 male, adult monkeys (*Macaca fascicularis*) of about 4 years of age, weighing approximately 4 to 6 kg were used in this study. Permission to carry out the study was granted by the Institutional Animal Care and Use Committee of SingHealth in Singapore.

The monkeys were fasted overnight and received 0.05mg/kg intravenous atropine and 10mg/kg ketamine preoperatively. Induction of anaesthesia was performed by a veterinarian using 3% halothane. Endotracheal intubation was done

using oral endotracheal tubes of gauge 3.5mm. The anaesthesia was maintained using 1-2% halothane. Intravenous analgesic 2mg/kg carprofen (Rimadyl®) and antibiotics 5mg/kg enrofloxacin (Baytril®) and 15mg/kg amoxicillin (Betamox®) were administered.

The upper left permanent central and lateral incisors (#21, 22) were extracted and a trapezoidal mucoperiosteal flap extending from the #21 to 22 region was raised. A bony defect was created by removing the facial wall of the #21, 22 tooth sockets using an osteotome.

Test group (N = 10): The PCL-TCP scaffold was shaped, using a no.15 surgical blade, to fit the #21, 22 tooth socket defect snugly. The implant site was prepared using successive drills as according to the Biomet 3i® (Biomet, Florida, USA) dental implant surgical protocol, at a drilling speed of 1200 rpm. Drilling was done through the PCL-TCP scaffold and into the apico-palatal bone of the tooth socket. The implant fixture was then inserted at insertion torque of 20 Ncm, through the PCL-TCP scaffold and into the apico-palatal bone (Figure 1a and 1b)

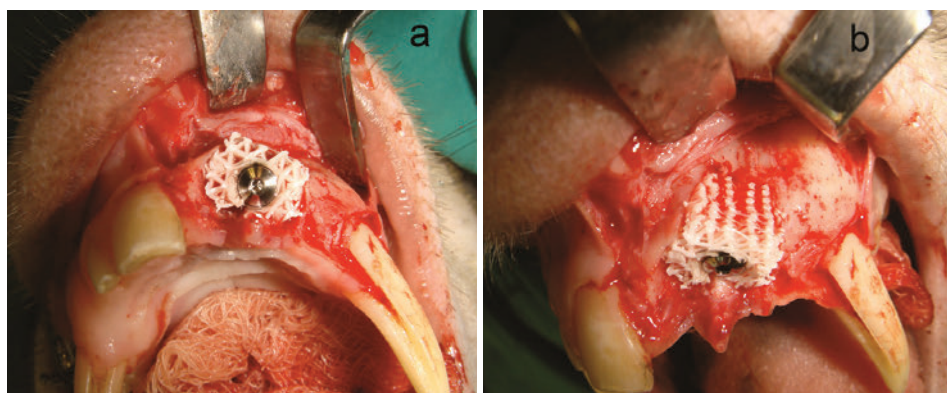


Figure 1. Test group – PCL-TCP scaffold and dental implant inserted into a tooth extraction site with a facial wall defect. **a)** Crestal view **b)** Facial view

Control group (N = 8): The implant site was prepared using successive drills as according to the Biomet 3i® (Biomet, Florida, USA) dental implant surgical protocol, at a drilling speed of 1200 rpm. Drilling was done directly into the apico-palatal part of the tooth socket. The implant fixture was then inserted at insertion torque of 20 Ncm into the apico-palatal bone. Bone from the previously removed facial plate of the tooth sockets in the same monkey was divided into smaller pieces with bone ronguers and the bone chips were grafted into the defect surrounding the dental implant (Figure 2a and 2b).

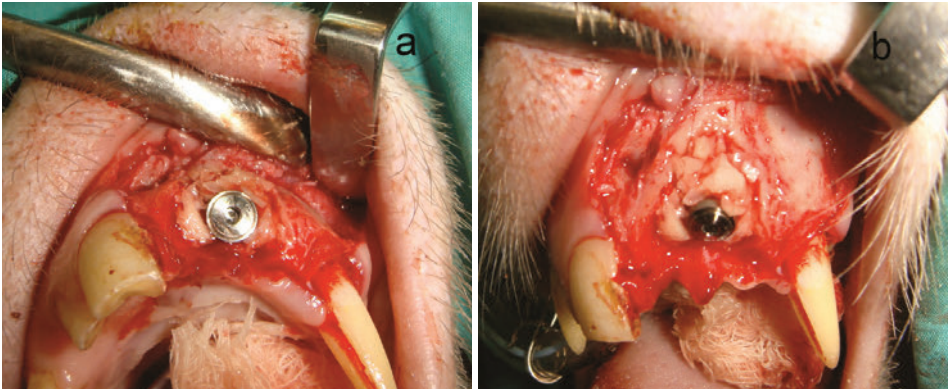


Figure 2. Control group – Autogenous particulate cortical bone grafted around a dental implant in a tooth extraction site with a facial wall defect. **a)** Crestal view **b)** Facial view

For both groups, the baseline Implant Stability Quotient (ISQ) was measured by Resonance Frequency Analysis (RFA) using the Osstell® machine in the bucco-lingual (B-L) and mesio-distal (M-D) directions immediately after implant insertion. The cover screw was then inserted. The periosteum of the labial flap was incised to achieve a tension-free primary closure of the surgical wound using 4/0 Vicryl® sutures. Immediately after the surgery, an anterior maxillary occlusal radiograph was taken using standardised settings. The monkeys were put on a soft diet for the first two weeks postoperatively. They received 2mg/kg Rimadyl® s.c., for analgesia for 2 days and 5mg/kg Baytril® and 15mg/kg amoxicillin s.c. for 5-7 days. The monkeys were sedated at 1 month and 3 month postoperatively for inspection of the surgical wounds.

Sacrifice

The monkeys in both the test and control groups were sacrificed at 6 months post-op. A perfusion fixation method was used. Before sacrifice, the operated site was examined for any signs of infection, soft tissue dehiscence and mobility of the scaffold and/or dental implant. The cover screw of the dental implant was then exposed using a tissue punch. ISQ was determined by RFA using the Osstell® machine in the B-L and M-D directions. After sacrifice, en-bloc resection of the #21, 22 alveolus, including the dental implant, was done. An anterior maxillary occlusal radiograph was taken of the specimen using the same settings as before. Any significant radiolucency or bone loss was noted, using the immediate post-op radiograph for comparison. The block was then sent for histologic preparation.

Histology and Histomorphometry

The tissue blocks were dehydrated in a graded series of ethanol, embedded in methyl methacrylate resin and polymerized. The tissue blocks with implants were mounted in a modified inner circular saw microtome (Leica® RM 2165, Wetzlar, Germany), and 10µm thick sections were prepared. Three longitudinal sections were made parallel to the long axis of the dental implant in each of 3 control and 6 test specimens. Three cross-sections were made perpendicular to the long axis of the dental implant at the crestal one-third level in each of the remaining 5 control and 4 test specimens. The sections were stained with methylene blue and basic fuchsin for histologic and histomorphometric analyses. A light microscope (Olympus® CX31, Tokyo, Japan) was used for histologic evaluation. The sections were examined for bone remodelling, bone regeneration and presence of any inflammatory reaction.

The following histomorphometric analyses were done:

1) Modified histologic grading scale for bone implants²⁸

A modified histologic grading scale was used (Table 2) to quantify the histologic findings of the reconstructed region surrounding the dental implants. Both the longitudinal and cross-sectional sections of the 8 control and 10 test specimens were evaluated. The grading was performed by 2 observers (GBT, NC) and when their grades differed, a consensus was reached after discussion.

Table 2. Modified histologic grading scale for bone implants

Evaluation	Response	Score
TEST GROUP: Interstitial Tissue	Mainly bone	4
	Fibrous tissue and some bone	3
	Mainly or only fibrous tissue with some inflammatory cells	2
	Only inflammatory cells	1
Bone-Scaffold interface	Bone contact + bone ingrowth in the scaffold porosities	3
	Fibrous tissue + no bone ingrowth	2
	Inflammatory cells	1
CONTROL GROUP: Particulate Bone Graft	Bone formation, defect completely closed	3
	Limited bone formation, defect partially filled, ingrowth of bone along implant surface	2
	No bone formation, collapse of gingiva, no particles left	1

2) Bone-to-implant contact (%BIC)

The %BIC was evaluated on the longitudinal sections made in 3 control and 6 test specimens. This was performed using the Cell A® (Olympus, Tokyo, Japan) imaging and histomorphometric software in a region of interest (ROI), defined as the labial surface of the dental implant, from the implant shoulder to the level where the implant inserted into native bone apically. It was calculated using the formula below:

$$\%BIC = \frac{[\text{total length of bone contact with implant surface in the ROI}]}{[\text{total length of implant surface in the ROI}]} \times 100$$

3) Bone area (%BA)

The %BA was also evaluated on the longitudinal sections made in 3 control and 6 test specimens. This was done using the Cell A® (Olympus, Tokyo, Japan) imaging and histomorphometric software in a ROI, defined as the region from 0 to 1mm labial to the trough of the implant threads (width) and from the implant shoulder to the level where the implant inserted into the native bone apically (length) (Figure 3). It was calculated using the formula below:

$$\%BA = \frac{[\text{total area of bone in the ROI}]}{[\text{total area in the ROI}]} \times 100$$



Figure 3. ROI for %BA measurement

Results

Clinical

All 18 monkeys were able to feed well orally and none had significant weight loss postoperatively. During wound inspection at 1 month, 1 animal (C1) in the control group presented with a small area of wound dehiscence at the crestal aspect resulting in an exposure of the implant cover screw. 1 animal (T6) in the test group presented with a relatively large area of wound dehiscence, exposing both the implant cover screw as well as the crestal one-third of the PCL-TCP scaffold. The exposed scaffold was contaminated with food debris but there was minimal swelling and no purulence noted in the surrounding tissues. The exposed dental implant and scaffold were completely removed under general anaesthetic. A new dental implant and PCL-TCP scaffold were inserted and the wound was closed primarily. Postoperatively, the monkey received s.c. analgesics and antibiotics as previously described. At the 3-month postoperative wound inspection, 4 of the 8 monkeys in the control group showed significant ridge resorption in the buccal aspect as the dark metallic color of the dental implant could be seen under the soft tissues (Figure 4a). The previously exposed implant cover screw in the control monkey remained similarly exposed. In all the test animals, the ridge contour appeared to be well maintained (Figure 4b). There was recurrence of wound dehiscence in the previously re-operated monkey but this time only the implant cover screw was exposed but not the scaffold. There was exposure of the implant cover screw and scaffold in another 2 test animals (T5 and T9). No active treatment was instituted in these 2 animals. The observational findings in all animals prior to sacrifice at 6 months were similar to those described at 3 months. There was no active infection or loss of the dental implant in any monkey throughout the duration of the study.

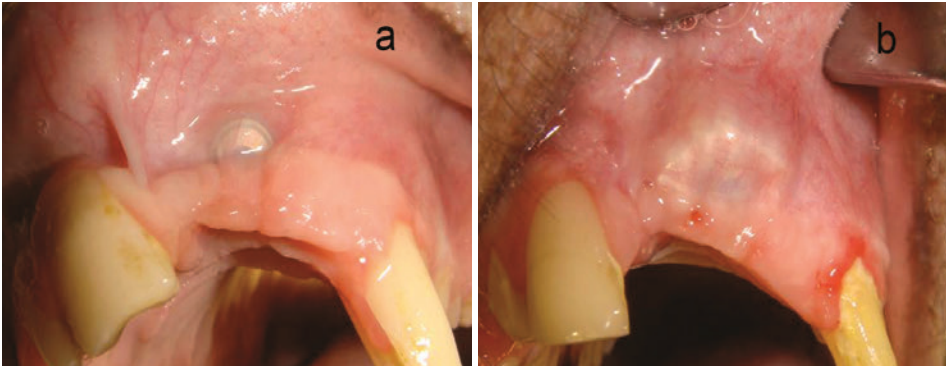


Figure 4. Alveolar ridge at 6 months. **a)** Control animal **b)** Test animal

Plain Radiographs

The anterior maxillary occlusal radiographs taken at sacrifice in both control and test animals did not show any significant radiolucencies, crestal bone loss or other abnormalities in the periimplant region. The dental implants did not show any signs of displacement or dislodgement. (Figure 5a and 5b)

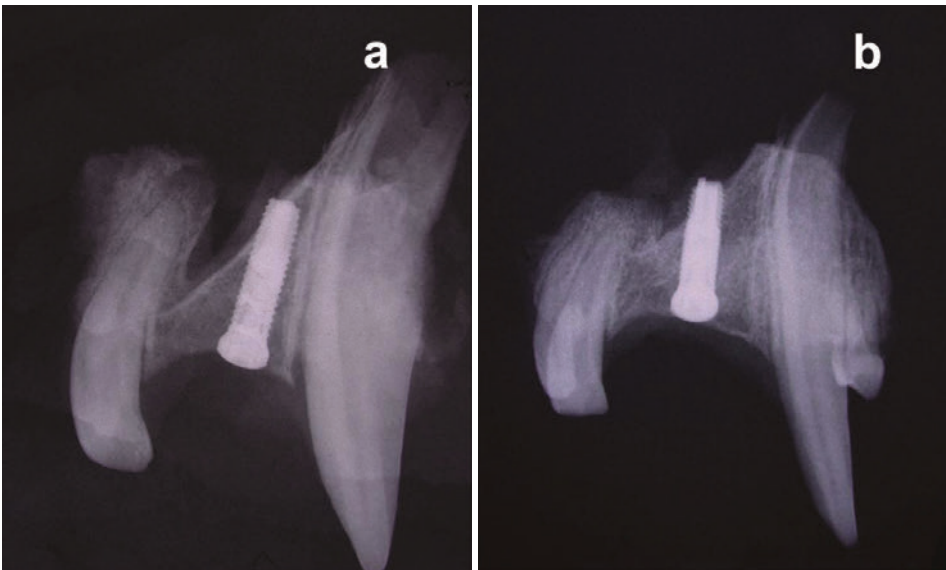


Figure 5. Anterior maxillary radiograph at 6 months (post-harvest).
a) Control animal **b)** Test animal

RFA

For the control group, the mean baseline ISQ values were 54 ± 6.9 (B-L) and 51 ± 8.7 (M-D), while the mean 6-month ISQ values were 60 ± 6.7 (B-L) and 64 ± 7.3 (M-D). For the test group, the mean baseline ISQ values were 51 ± 6.6 (B-L) and 49 ± 5.4 (M-D), while the mean 6-month ISQ values were 54 ± 14.4 (B-L) and 55 ± 15.5 (M-D). Although the mean ISQ values at 6 months seemed higher than at baseline for both the control and test groups, this could not be proven by formal statistical tests due to small sample sizes. (Figure 6)

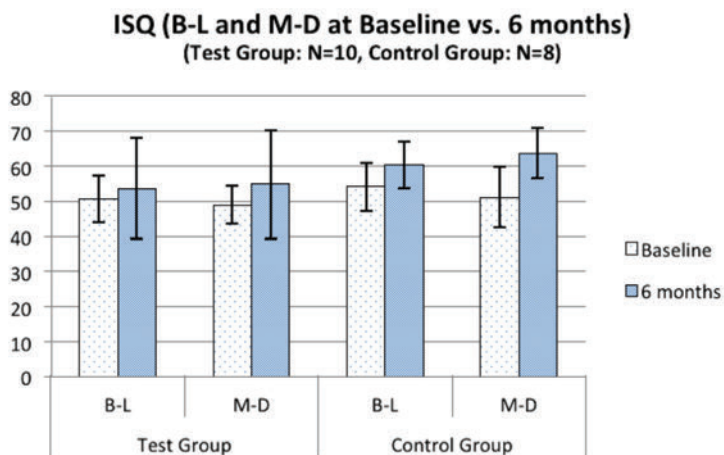


Figure 6. ISQ values at baseline and 6 months of test and control groups

Descriptive Histology

Longitudinal Sections

In all the longitudinal sections, the dental implant was seen to be inserted into native alveolar bone at its apical third and in a few specimens, it penetrated through the bony nasal floor into the nasal cavity. Crestally, the gingival tissue overlying the cover screw was missing as it had been removed just before the euthanasia so that the RFA could be performed.

In the control specimens, native alveolar bone and new bone were seen in direct contact with at least the apical two-thirds of the implant surface on the palatal aspect. The labial surface of the dental implant was covered by labial muscle and mucosa, with a total absence of bone (Figure 7). In the test specimens, the PCL-TCP scaffold was seen as either brownish-grey concentric areas or empty concentric spaces as the scaffold was partially dissolved during the histologic processing. A greater thickness

of the PCL-TCP scaffold was always seen on the labial than the palatal aspect of the implant. On the palatal aspect, the scaffold appeared compressed due to the dental implant installation (Figure 8a). The porosities of the scaffold were mostly occupied by fibrous tissue with occasional islands of new bone just adjacent to the native palatal or apical alveolus (Figure 8b). There was almost an absence of inflammatory cells within the fibrous infiltrate. A ring of inflammatory cells, however, was often seen encircling the PCL-TCP struts (Figure 8c). In those specimens where there was soft tissue dehiscence clinically (T5, T6 and T9), abundant inflammatory cells were seen infiltrating the porosities of the scaffold.

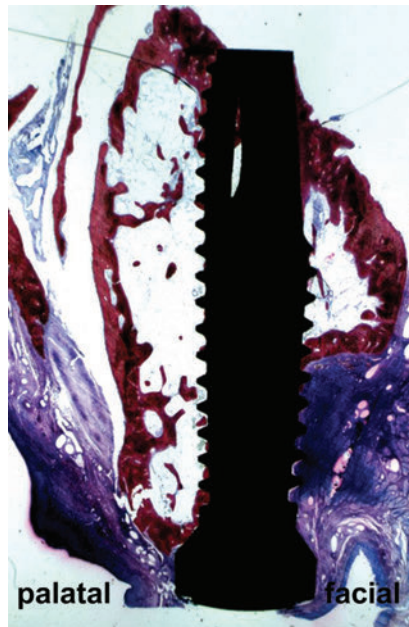


Figure 7. Control specimen longitudinal section (methylene blue and basic fuchsin stain)

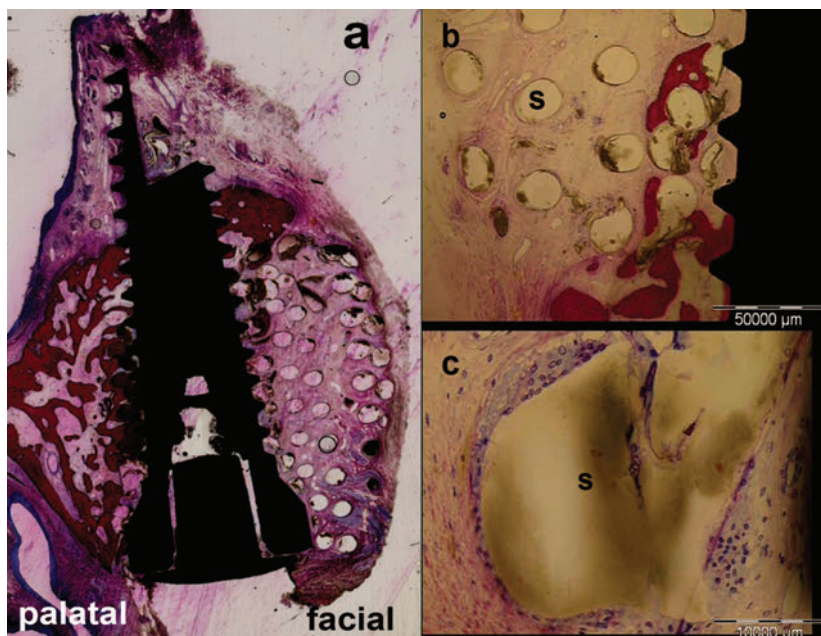


Figure 8a, b and c. Test specimen longitudinal section (methylene blue and basic fuschin stain) S: PCL-TCP scaffold

Cross-Sectional Sections

The cross-sectional sections were made at the crestal third level of the dental implants so as to avoid sectioning through the apical native alveolar bone. In the control specimens, bone was seen on the palatal, mesial and distal aspects, contacting the dental implant surface. On the labial aspect, the implant surface was covered by fibrous tissue and labial muscle in 3 out of 4 specimens (Figure 9). In 1 specimen, new bone was seen covering and in contact with the labial aspect of the dental implant. In the test specimens, the PCL-TCP scaffold was seen to surround the dental implant. Again, the scaffold was thicker on the labial than the palatal aspect. Similar to the longitudinal sections, fibrous tissue was seen predominantly within the scaffold porosities with minimal inflammatory cells, except for the specimens that had soft tissue dehiscence. Labially, a fibrous capsule surrounded the scaffold. Small islands of bone were seen growing into the porosities of the scaffold just adjacent to the palatal native bone. (Figure 10)

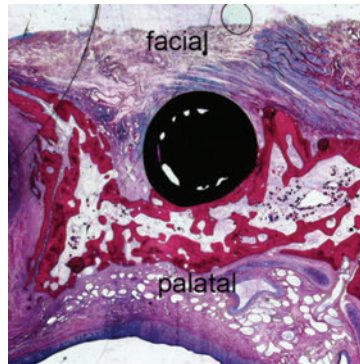


Figure 9. Control specimen cross-sectional section (methylene blue and basic fuchsin stain)

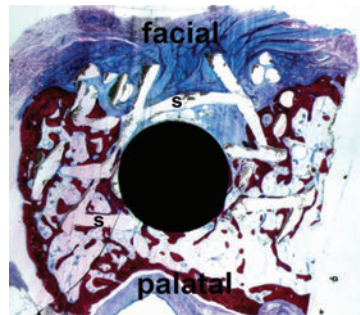


Figure 10. Test specimen cross-sectional section (methylene blue and basic fuchsin stain)

Histomorphometry

1) Modified histologic grading scale for bone implants

For the control group, the mean score for the particulate bone graft was 1.33 ± 0.69 . Only 1 specimen scored 3 for all sections while 5 specimens scored 1 for all sections.

For the test group, the mean score was 2.62 ± 0.56 for interstitial tissue. None of the sections scored 4. Most of the sections scored either 2 or 3, except for those sections belonging to animals T5 and T6, which scored 1. The mean score was 2.28 ± 0.74 for bone – scaffold interface. Likewise, most sections scored either 2 or 3 with the exception of 1 section belonging to animal T6, which scored 1. (Figure 11)

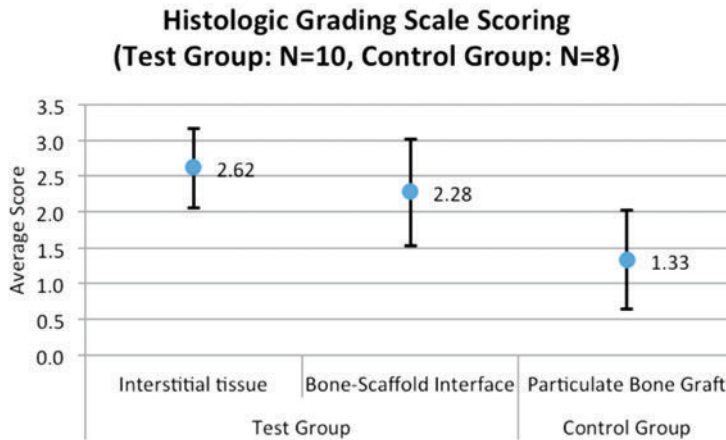


Figure 11. Histologic grading scale scores of test and control groups

2) %BIC

The mean %BIC was $27.6 \pm 19.1\%$ for the control group and $6.8 \pm 7.9\%$ for the test group. Two out of the 6 test specimens had zero %BIC (T2 and T6). Formal statistical tests could not be performed due to small sample sizes. (Figure 12)

3) %BA

The mean %BA was $11.8 \pm 10.1\%$ for the control group and $6.8 \pm 6.9\%$ for the test group. One specimen in the test group had zero %BA (T6). Formal statistical tests could not be performed due to small sample sizes. (Figure 12)

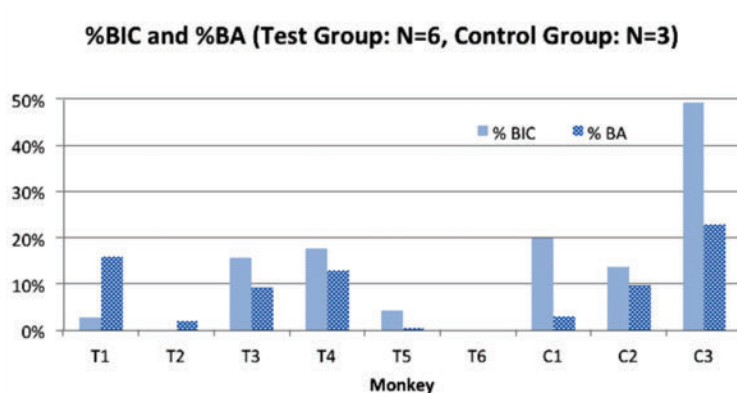


Figure 12. %BIC and %BA of test and control animals

Discussion

The present study used a clinically relevant model to study the feasibility of using a PCL-TCP scaffold for reconstruction of a socket wall defect. In addition, it tested if dental implant placement through the scaffold, done at the same surgery, was technically possible. Combining the bone regenerative procedure with the dental implant placement not only reduced the number of surgeries and total treatment time, the dental implant also served as a fixation device to immobilize the scaffold. The monkey model was selected because of the phylogenetic relationship with human. The monkey is omnivorous and is considered to best represent the human masticatory system²⁹.

The PCL-TCP scaffold used in the study was prefabricated in the form of a standardized cuboidal block. During surgery, it could be easily shaped using a scalpel so as to fit the size and shape of the tooth extraction site defect. It was also noted during the study that the PCL-TCP scaffold possessed sufficient mechanical strength to withstand drilling with twist drills, without undergoing gross deformation or disintegration. Experience from this study therefore demonstrated that customization of the PCL-TCP scaffold would not be necessary for this clinical application. This would save production cost and time. When manually shaping the scaffold, however, it is important to ensure that the scaffold is not over-contoured. In this study, 3 test animals presented with wound dehiscence that resulted in the exposure of the scaffold and implant cover screw. It was likely that the scaffold was over-contoured and the wound closure was not completely tension-free in these animals. It is important to note that while healing by secondary intention frequently occurs when an autogenous bone graft is exposed, when a porous scaffold is exposed, it becomes a trap for food and oral microorganisms and healing becomes difficult or impossible. In this study, the histological sections of those animals that had soft tissue dehiscence showed an abundance of inflammatory cells and minimal or no bone growth.

Four of the 8 animals in the control group showed significant resorption at the grafted site that was noted clinically as early as 3 months post-op. This observation was confirmed histologically, where there was a collapse of soft tissue into the defect and a complete absence of bone on the labial aspect. Our findings in the control group were consistent with those reported by Schliephake *et al*³⁰ in a study that evaluated the use of autogenous particulate bone, either with or without a barrier membrane for the repair of alveolar ridge defects and simultaneous implant placement in a dog model. They found only a minor increase in bone

height using these augmentation techniques. The major limiting factor for bone regeneration appeared to be insufficient stability of the bone graft to withstand the overlying soft tissue pressure. It was concluded in that study that the placement of autogenous bone particles had little effect on the regeneration of peri-implant bone defects. Although autogenous bone is still considered the gold standard as a graft material due to its supply of osteoprogenitor cells, it is a well-known fact that over time, it undergoes variable but often substantial resorption. The physiology of particulate bone graft is explained by the Axhausen theory of osteogenesis³¹ which states that surviving transplanted cells proliferate and form new random osteoid, which is dependent on the spatial orientation of the grafted tissue. Thus, the eventual quantity of bone is determined in Phase I. Phase II of osteogenesis results in resorption and remodeling of the random osteoid into mature osteons with organized structures. The quality of bone is, thus, determined in Phase II. The process of replacement of the immature Phase I bone with organized Phase II bone is dependent on the volume and cellularity of the host bone surrounding the defect as well as the supply of mesenchymal cells derived from the overlying soft tissues. It has been suggested that the reduced volume and cellularity of the bone at the margins of a facial bone defect is insufficient to maintain the graft volume during Phase II osteogenesis resulting in loss of graft volume over time.¹¹ While it is questionable whether complete facial bone coverage of the implant surface is necessary for implant survival, the loss of labial ridge contour, soft tissue recession and exposure of the underlying metal implant are major concerns especially in the anterior aesthetic zone.

In this study, the PCL-TCP scaffold was able to maintain the labial contour of the alveolar ridge in the test animals over the 6-month study duration. This could be attributed to the mechanical stability, structural integrity and relatively slow degradation rate of the PCL-TCP scaffold. It is a bioengineering challenge to fabricate a scaffold with the ideal degradation profile that matches the rate of new bone formation. In principle, when implanted in the body, a bioresorbable scaffold degrades and is absorbed by the body over time, enabling the space occupied by the scaffold to be gradually replaced by newly formed bone.³² If the scaffold degrades too early, before sufficient bone ingrowth and consolidation can occur, it might not be able to withstand the load bearing forces that are present in the oral cavity. Conversely, if the scaffold takes too long to degrade, it will act as a barrier and hinder new bone formation within the defect. PCL, like other members of the aliphatic polyesters, undergoes a 2-step degradation process. The first step occurs by autocatalysis with non-enzymatic random hydrolytic chain scission of ester linkages.

The second step occurs as the mechanical strength and weights are lost, thereby increasing the surface area for bio-erosion. The final breakdown products of PCL are CO_2 and H_2O .¹³ PCL scaffolds degrade at a slow rate due to its high molecular weight and hydrophobicity. The incorporation of TCP into a PCL polymer matrix produces a hybrid or composite material that allows tailoring the desired degradation kinetics of the polymer matrix. The added TCP particles are physically blended into the polymer and occupy random spaces in the polymer. Shortly after the scaffold is implanted in the body, the TCP particles, being hydrophilic, tend to fall off and interact with the surrounding body fluid. The dislodgement of TCP creates voids within the polymer, thus exposing their surfaces to hydrolytic attack and weakening the overall structure of the PCL.²¹ For dento-alveolar reconstruction, a scaffold that degrades in about 5 to 6 months is considered ideal for bone regeneration and remodeling.²⁴ In this study, it was noted histologically that much of the scaffold was still present at 6 months. Minimal bone ingrowth was noted only in those areas where the scaffold was in contact with a bony wall of the socket. On the labial aspect, in the absence of a bony wall, there was a lack of bone regeneration. Instead, fibroblasts from the labial soft tissue flap grew and proliferated in the porosities of the scaffold. It can therefore be anticipated that over time, as the scaffold continues to degrade and lose its structural form, in the absence of new bone growth on the labial aspect, the alveolar contour will correspondingly collapse.

Dental implant survival rate was 100% in both the test and control groups at 6 months. No drastic drop in ISQ was noted in any of the animals at 6 months, therefore supporting the fact that there was no loss of implant stability.

In conclusion, although a 3D bioresorbable PCL-TCP scaffold, when employed for the reconstruction of a facial wall defect during immediate dental implant placement, showed better maintenance of the alveolar contour as compared to the use of autogenous particulate bone at 6 months, only minimal bone regeneration and bone-to-implant contact were noted in areas directly adjacent to a bony wall of the defect. The hypothesis for this study therefore, could not be supported.

Future studies shall explore the use of a barrier membrane to seclude the scaffold area from connective tissue cells and/or the use of growth factors to enhance osteogenesis within the scaffold. Further work shall also be done to treat or modify the PCL-TCP scaffold so as to achieve a more favorable degradation time of 5 to 6 months.

Acknowledgements

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Conflict of Interest

Co-author Swee Hin Teoh is President and Chairman Board of Directors of Osteopore International (Singapore) that manufactures and markets the PCL-TCP scaffold used in this study.

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CHAPTER 7

SUMMARY, CLOSING REMARKS AND FUTURE PERSPECTIVE

Summary

This thesis aims to investigate novel methods of oral and maxillofacial bone reconstruction that may potentially avoid the need to harvest bone from a donor site.

In particular, it focuses on further development of the mandibular endoprosthesis for use in the reconstruction of a mandibular body segmental defect. The current thesis investigates the use of a regenerative medicine approach to favor the bone as well as soft tissue response to a mandibular endoprosthesis. In **Chapter 1**, a general introduction on the mandibular endoprosthesis and regenerative medicine as well as a description of the aims of this thesis are presented.

Although the initial results with the endoprosthesis are promising, there are some issues dealing with the use of the PMMA cement as well as the final tissue responses (soft and hard tissue) to the body component of the device. Recently, advancements have been made in a new field of “regenerative medicine”. In regenerative medicine, biomolecules and stem cells are used to enhance tissue regeneration. In addition, the biomaterial (scaffold), which is used as a carrier for the biomolecules and stem cells, is newly designed so that it is able to direct the organization, growth and differentiation of the forming functional tissue. Despite significant surgical advances over the last decades, segmental mandibular bone repair remains a challenge. In light of this, tissue engineering may offer a next step in the evolution of mandibular reconstruction. Therefore, the purpose of **Chapter 2** was (1) to systematically review preclinical *in vivo* as well as clinical literature regarding bone tissue engineering for mandibular continuity defects, and (2) to analyze their effectiveness. An electronic search in the databases of the National Library of Medicine and ISI Web of Knowledge was carried out. Only publications in English were considered and the search was broadened to animals and humans. Furthermore, the reference lists of related review articles and publications selected for inclusion in this review were systematically screened. Results of histology data and amount of bone bridging were chosen as primary outcome variables. However, for human reports, clinical radiographic evidence was accepted for defined primary outcome variable. The biomechanical properties, scaffold degradation as well as clinical wound healing were selected as co-outcome variables. The electronic search in the databases of the National Library of Medicine and ISI Web of Knowledge resulted in the identification of 6727 and 5017 titles respectively. Thereafter, title assessment and hand search resulted in 128 abstracts, 101 full-text articles and 29 scientific papers reporting on animal experiments as well as 11 papers presenting

human data on the subject of tissue engineered reconstruction of mandibular continuity defects that could be included in the present review. On the basis of these findings, it was concluded that: (1) published preclinical in vivo as well as clinical data are limited, and (2) tissue engineered approaches demonstrate some clinical potential as an alternative to autogenous bone grafting.

The aim of the study as described in **Chapter 3** was to evaluate the soft tissue response to poly(ϵ -caprolactone) (PCL) implants with and without carbonate-substituted hydroxyapatite (CHA) coating compared to the commonly used titanium alloy (Ti-6Al-4V)-machined surface. Experimental materials were implanted subcutaneously in New Zealand white rabbits for 5 weeks. The tissue attachment strength, as evaluated by a tissue peel test, histological and histomorphology analysis as well as scanning electron microscopy (SEM) were compared between groups. The peel test result revealed no statistically significant difference between groups. Histological analysis found fibrous capsule formation around all implant materials. The fibrous capsule around PCL implants with and without CHA coating was significantly thinner compared with the capsule thickness around the titanium implants. However, the inflammatory cells, as present at the fibrous capsule-implant interface, were found to be significantly lower in Ti- group. In conclusion, the current data do not prove that PCL or PCL with a CHA coating results in a superior soft tissue response compared with a machined titanium implant.

The study, as reported in **Chapter 4**, was designed to evaluate the effectiveness of bioactive-coated cementless modular mandibular endoprosthesis for mandibular reconstruction in *Macaca fascicularis*. The mandibular endoprosthesis was first introduced in 2008, composed of a body part and two stem components assembled together and of which the stem parts were inserted in the cancellous bone of the remaining stumps. These stems were fixed with polymethylmethacrylate (PMMA) cement. In this current thesis, we designed and explored the cementless mandibular modular endoprosthesis devices. The devices were implanted for 6 months at unilateral mandibular body segmental defects in 9 monkeys. Analysis was performed using biomechanical testing, histological and Micro CT analysis. Mandibular contour and initial stability were satisfactory at 6 months post-operation. Mechanical load bearing test showed mean stiffness value of 110.43 N/mm. Histomorphology analysis found 64.17% bone to stem contact. There was fibrous capsule and woven bone around the device body. Percent bone volume calculated from Micro-CT around the stem surface was found to be superior to previously reported cemented mandibular endoprosthesis. However, intraoral

wound dehiscence was found in 6 animals. This newly designed cementless mandibular endoprosthesis is feasible in mandibular segmental reconstruction. The intermodular connection screw loosening has been resolved compared to the previous model. However, insufficient load-bearing capability and improper soft tissue healing were found in majority of the animals. Further modifications to the device and surgical technique need to be addressed in future studies.

In **Chapter 5**, a study was presented, which was aimed to evaluate the regeneration of a segmental mandibular bone defect by means of a 3-D designed PCL scaffold provided with a carbonate-substituted hydroxyapatite (CHA) coating for the delivery of osteoinductive factors to the defect site. The study was performed in a non-human primate model, *Macaca fascicularis* using an engineered poly(ϵ -caprolactone) (PCL) scaffold, provided with a carbonate substituted hydroxyapatite (CHA) coating. The scaffolds were implanted in unilateral surgically created mandibular segmental defects in 24 monkeys. Three experimental groups were made; (1) scaffolds with rhBMP-2 (n=8), (2) scaffolds with autologous mixed bone marrow cells (n=8), and (3) empty scaffolds as a control group (n=8). Evaluation was based on clinical observation as well as micro-CT, mechanical and histological analyses. Apart from a high infection rate, the results showed that the newly designed PCL scaffold had insufficient load-bearing capability and a complete bony union could not be achieved after 6 months of implantation. Nevertheless, the group that was implanted with PCL scaffold loaded with rhBMP-2 showed evidence of bone regenerative potential as compared to the group implanted with PCL and autologous mixed bone marrow cells and the control group.

The last study, as described in **Chapter 6**, was focused on peri-implant bone regeneration and implant stability following immediate implant placement into tooth sockets with facial wall defects in 2 treatment groups. In 8 monkeys, the bone defect was reconstructed with autogenous particulate bone, while in 10 other monkeys an experimental PCL-TCP scaffold was used. The monkeys were sacrificed after 6 months and the specimens were analyzed by histology and histomorphometry. Better maintenance of facial bone contour was noted in the experimental group, however bone regeneration was seen only at areas adjacent to a bony wall of the defect. The mean bone-to-implant contact was $27.6 \pm 19.1\%$ (control group) versus $6.8 \pm 7.9\%$ (experimental group). The mean bone area percentage was $11.8 \pm 10.1\%$ (control group) versus $6.8 \pm 6.9\%$ (experimental group). Implant survival was 100% at 6 months for both groups. It was concluded that although the use of a PCL-TCP scaffold showed better maintenance of the

alveolar contour as compared to autogenous particulate bone at 6 months, there was minimal bone regeneration within the defect.

Closing Remarks and Future Perspectives

The results of this thesis have shown that alternative methods to the use of autogenous bone for oral and maxillofacial bone reconstruction are promising, although several drawbacks in the design of the modular endoprosthesis and tissue engineering techniques need to be addressed.

The metallic bioactive-coated cementless modular endoprosthesis design reported in this thesis for reconstruction of mandibular segmental defects is an improvement to the previously reported cemented device. This is based on the study findings of increased bone-to-stem contact and that the screw-less intermodular locking mechanism seems to have resolved the problem of component loosening. A screw-less locking design should therefore be adopted in future modifications of the device. Problems that persisted even in this cementless endoprosthesis are soft tissue dehiscence and insufficient load-bearing capability. These problems may be partly due to the use of an animal model where it is impossible to apply intermaxillary fixation in the immediate postoperative period and that the monkeys are able to disturb the surgical wound with their fingers. In future studies, the use of an alternative animal model e.g. goat or sheep and an extra-oral surgical approach will be considered. As with dental implants, a period of healing to allow for osseointegration before loading the cementless device should be considered for future experiments. To achieve this, the rigid interlocking of the modules may have to be delayed and performed at a second surgery several months later. By doing this, the stems of the device will be spared from loading during the early healing period. The search for an ideal surface modification or coating that will enhance soft tissue adhesion to the metal surface should also continue.

Tissue engineering methods to grow the patient's own bone, if successful, will undoubtedly be the ideal solution for bone reconstruction. Although many successful reports of bone engineering are available in *in vitro* and small animal models, reports of clinical success are scarce. The less positive results in clinical cases are due to various factors including insufficient vascularization of the tissue-engineered construct, compromised soft tissue bed and biomechanical factors.

In this thesis, bone tissue engineering was tested for regeneration of a small sized alveolar defect and also for a large sized mandibular segmental defect that was subjected to functional loading. From these studies, it seems that the PCL scaffold,

whether incorporated with TCP or coated with CHA, is not osteoinductive. The ideal scaffold material should have a resorption time that matches the rate of new bone ingrowth. As the PCL composite scaffold may be taking too long to resorb, it is treated as a foreign material by the body resulting in fibrous tissue invasion. Research should therefore be done to modify or treat the PCL scaffold so as to achieve a more ideal resorption time.

For mandibular reconstruction, the PCL scaffold was shown to have insufficient load-bearing capability. There was a high rate of infection and dislodgement of the fixation plates and the PCL endoprosthesis scaffold. Nevertheless, the BMP-2-loaded PCL scaffolds were found to perform better in terms of bone formation and mechanical testing than empty PCL scaffolds and scaffolds loaded with autogenous BMSCs. However, there is an increasing number of reports e.g. Perri *et al.*, 2007, Shah *et al.*, 2008 and Carragee *et al.*, 2013, which describe that the clinical use of BMP-2 for bone regeneration is associated with massive soft tissue swelling postoperatively, ectopic bone formation and potential oncogenic effects. These complications, in addition to its high cost may limit its routine clinical use. Currently, the Centre for Devices and Radiological Health (CDRH) of the U.S. Food and Drug Administration (FDA) has not licensed rhBMP-2 for use in mandibular continuity defects and therefore this application will be considered “off-label”.

Research to improve the success of bone tissue engineering in the clinical setting should therefore continue. For a load-bearing, large size defect such as the mandible, particular attention should be given to evaluation of the complex biomechanical forces. Microvascular anastomosis may still be necessary in combination with bone tissue engineering methods to ensure adequate vascularization of the entire construct.

**SAMENVATTING,
AFSLUITENDE OPMERKINGEN
EN TOEKOMSTPERSPECTIEF**

Samenvatting

Dit proefschrift wil nieuwe methoden van orale en maxillofaciale botreconstructie onderzoeken waardoor het misschien niet meer nodig zal zijn om bot uit een donorsite weg te nemen.

De aandacht gaat met name uit naar de verdere ontwikkeling van de mandibulaire endoprothese voor gebruik bij de reconstructie van een mandibulair segmentaal defect in het lichaam. In de diverse studies wordt het gebruik van regeneratieve geneeskunde voor een betere respons van zowel het bot als de weke delen op een mandibulaire endoprothese onderzocht. In **Hoofdstuk 1** wordt er een algemene inleiding gegeven over de mandibulaire endoprothese en de regeneratieve geneeskunde, en worden ook de doelstellingen van dit proefschrift beschreven.

Ondanks de veelbelovende resultaten met de endoprothese, zijn er toch wel een aantal problemen met het gebruik van het PMMA-cement en met de uiteindelijke respons van de weefsels (zowel de weke delen als het harde weefsel) op dit implantaat. Onlangs werd er vooruitgang geboekt in het nieuwe wetenschapsgebied van de 'regeneratieve geneeskunde'. In de regeneratieve geneeskunde wordt er gebruik gemaakt van biomoleculen en stamcellen om de regeneratie van weefsel te stimuleren. Bovendien is het biomateriaal (scaffold), dat gebruikt wordt om de biomoleculen en stamcellen te transporteren, zodanig ontworpen dat het de structuur, groei en differentiatie van het vormende functionele weefsel kan sturen. Hoewel er de laatste decennia op chirurgisch vlak heel wat vooruitgang is geboekt, blijft segmentaal mandibulair botherstel een uitdaging. In het licht hiervan kan weefselengineering een volgende stap betekenen in de evolutie van mandibulaire reconstructie. Allereerst hebben wij **Hoofdstuk 2** (1) systematisch de preklinische *in vivo* en klinische literatuur over botweefselengineering voor mandibulaire continuïteitsdefecten bestudeert en (2) vervolgens de doeltreffendheid hiervan geanalyseerd. Er werd elektronisch opzoekingswerk verricht met behulp van de databanken van de National Library of Medicine en ISI Web of Knowledge. Enkel publicaties in het Engels werden in aanmerking genomen en de opzoeking werd uitgebreid tot mensen en dieren. Bovendien werden de referentielijsten van aanverwante recensieartikelen en publicaties, die geselecteerd werden om in deze beoordeling te worden opgenomen, systematisch gescreend. De resultaten van histologische gegevens en de hoeveelheid botoverbrugging werden gekozen als primaire uitkomstvariabelen. Voor verslagen over mensen werd echter klinisch röntgenologisch bewijs aanvaard als een gedefinieerde primaire uitkomstvariabele. De biomechanische eigenschappen, de afbraak van de scaffold en de klinische

wondheling werden gekozen als co-uitkomstvariabelen. De elektronische opzoeking in de databanken van de National Library of Medicine en ISI Web of Knowledge leidde tot de identificatie van respectievelijk 6727 en 5017 titels. Uiteindelijk resulteerde de beoordeling van de titels en het handmatige zoeken in de inclusief van 128 samenvattingen, 101 volledige tekstartikelen en 29 wetenschappelijke papers die betrekking hadden op dierstudies, en 11 humane studies waar mandibulaire continuïteitsdefecten gereconstrueerd waren met gekweekt weefsel. Op basis van deze bevindingen werd het volgende geconcludeerd, dat: (1) er zijn slechts beperkte gepubliceerde preklinische in vivo en klinische gegevens, en (2) weefselengineering toont klinisch potentieel als een alternatief voor autologe bottransplantatie.

Het doel van het onderzoek zoals beschreven in **Hoofdstuk 3** was het evalueren van de respons van de weke delen op poly(ϵ -caprolacton) (PCL) implantaten met en zonder carbonaatvervangende hydroxyapatiet (CHA) laag in vergelijking met de vaak gebruikte machinaal bewerkte titaniumlegering (Ti-6Al-4V). De experimentele materialen werden gedurende vijf weken subcutaan geïmplantatoed in Nieuw-Zeelandse witte konijnen. De sterkte van de weefselaanhechting, zoals beoordeeld aan de hand van een weefselpeltest, histologische en histomorfologische analyse, en scanning elektronenmicroscopie (SEM), werd vergeleken. De peltest liet geen statistisch significant verschil zien tussen de groepen. De histologische analyse toonde de vorming van een fibreus kapsel rond alle implantaten aan. Het fibreuze kapsel rond de PCL implantaten met en zonder CHA laag was aanzienlijk dunner dan het kapsel rond de titaniumimplantaten. Het aantal ontstekingscellen, die op het raakvlak van het implantaat met het fibreuze kapsel terug te vinden waren, was echter aanzienlijk lager in de Ti-groep. Er kan hieruit geconcludeerd worden dat de huidige gegevens niet aantonen dat PCL of PCL met een CHA laag resulteert in een betere respons van de weke delen in vergelijking met een machinaal bewerkte titaniumimplantaat.

Het onderzoek, zoals gerapporteerd in **Hoofdstuk 4**, was opgezet om de doeltreffendheid te evalueren van een cementloze modulaire mandibulaire endoprothese welke voorzien was van een bioactieve deklaag. De studie werd uitgevoerd in *Macaca fascicularis*. De mandibulaire endoprothese werd in 2008 voor het eerst geïntroduceerd. Ze bestond uit een dik middengedeelte en twee dunne stelen die worden samengevoegd en waarvan de stelen in het poreuze bot van de resterende botstompen worden ingebracht. Deze stelen worden vastgezet met PMMA-cement (polymethylmethacrylaat). In dit proefschrift hebben we gebruik gemaakt van een cementloze mandibulaire modulaire endoprothese. Deze endoprothese werden gedurende 6 maanden unilateraal geïmplantatoed in

mandibulaire segmentale defecten bij 9 apen. Na opoffering werd biomechanisch, histologische en micro-CT-analyse uitgevoerd. Zes maanden na de operatie waren de mandibulaire omtrek en de aanvankelijke stabiliteit bevredigend. De mechanische belastingsproef toonde een gemiddelde stijfheidswaarde van 110,43 N/mm. Uit de histomorfologische analyse bleek dat er 61,47% bot contact te zij met de steel van de endoprothese. Rondom het implantaat was een fibreus kapsel en trabeculair bot aanwezig. Het percentage botvolume berekend op basis van micro-CT rond het steeloppervlak bleek groter te zijn dan bij de eerder gerapporteerde gecementeerde mandibulaire endoprothese. Bij 6 dieren werd er echter intraorale wonddehiscentie aangetroffen. Deze nieuw ontwikkelde cementloze mandibulaire endoprothese is bruikbaar in geval van een mandibulaire segmentale reconstructie. In tegenstelling tot het vorige model komen de schroeven waarmee de verschillende modules met elkaar worden verbonden niet meer los. De weerstand tegen belasting en de genezing van de weke delen is echter nog niet optimaal. In toekomstig onderzoek moeten niet alleen de endoprothesen zelf verder worden aangepast, maar ook de gebruikte chirurgische techniek.

In **Hoofdstuk 5** is een onderzoek beschreven waarbij een segmentaal mandibulair botdefect werd gereconstrueerd d.m.v. een driedimensionaal ontwikkelde poly(ϵ -caprolacton) (PCL) scaffold met een carbonaatvervangende hydroxyapatiet (CHA) laag voor het afleveren van osteoinductieve factoren op de plaats van het defect. Het onderzoek werd uitgevoerd bij een niet-menselijke primaat, *Macaca fascicularis*. De scaffolds werden bij 24 apen ingeplant in unilaterale chirurgisch gecreëerde mandibulaire segmentale defecten. Er werden drie experimentele groepen gevormd: (1) scaffolds geladen met rhBMP-2 (n=8), (2) scaffolds geladen met autologe beenmergcellen (n=8) en (3) lege scaffolds als controlegroep (n=8). De evaluatie was gebaseerd op klinische observatie, en micro-CT-, mechanische en histologische analyse. Naast een hoog infectiepercentage toonden de resultaten ook aan dat er de ontwikkelde PCL scaffold in onvoldoende mate belasting man weerstaan. Zes maanden na de implantatie was er ook nog steeds geen volledige botverbinding. De meeste botvorming werd waargenomen in de PCL scaffold welke geladen waren met rhBMP-2.

Het laatste onderzoek, beschreven in **Hoofdstuk 6**, was toegespitst op botregeneratie rond een tandimplantaat dat wordt geplaatst in een implantaatbed, waarvan de botwand deels verdwenen is. De implantaten werden geplaatst bij apen, waarbij in 8 apen het botdefect werd gereconstrueerd met autologe botdeeltjes, terwijl bij 10 andere apen de experimentele PCL-TCP scaffold werd gebruikt. Na 6 maanden werden de apen opgeofferd en de weefselmonsters geanalyseerd

door middel van histologie en histomorfometrie. Na 6 maanden waren alle implantaten in beide groepen nog steeds aanwezig. In de experimentele groep werd opgemerkt dat de de contour van de processus alveolaris werd gehandhaafd, maar botregeneratie werd alleen waargenomen in het gebied dat vlak aan de rand van het defect grensde. Het gemiddelde contact tussen bot en implantaat bedroeg $27,6 \pm 19,1$ % (controlegroep) versus $6,8 \pm 7,9$ % (experimentele groep). De gemiddelde hoeveelheid bot in het defectgebied bedroeg $11,8 \pm 10,1$ % (controlegroep) versus $6,8 \pm 6,9$ % (experimentele groep). Er werd geconcludeerd dat, hoewel het gebruik van een PCL-TCP scaffold een betere handhaving aantoonde van de alveolaire contour in vergelijking met autologe botdeeltjes, er slechts sprake was van een minimale botregeneratie in het defect.

Slotopmerkingen en Toekomstige Vooruitzichten

Uit de resultaten van dit proefschrift blijkt dat alternatieve methoden voor het gebruik van autoloog bot voor orale en maxillofaciale botreconstructie veelbelovend zijn, hoewel het ontwerp van de modulaire endoprothese en de weefsel engineeringtechnieken op bepaalde vlakken toch wel moeten worden aangepast.

Het metalen cementloze modulaire ontwerp van de endoprothese met bioactieve laag die in dit proefschrift wordt besproken voor de reconstructie van mandibulaire segmentale defecten is een verbetering van de gecementeerde prothese. Dit is gebaseerd op de onderzoeksbevindingen, waarbij meer botcontact met de implantaatsteel werd waargenomen en het feit dat het nieuwe mechanisme om de verschillende modules vast te zetten het probleem van de loskomende onderdelen blijkbaar heeft opgelost. De problemen die zelfs met deze cementloze endoprothese blijven bestaan, zijn dehiscentie van de weke delen en een onvoldoende vermogen om belasting te weerstaan. Deze problemen kunnen deels te wijten zijn aan het gebruik van het huidige proefdiermodel, waarbij intermaxillaire fixatie onmiddellijk na de operatie onmogelijk is, maar ook aan het feit dat de apen met hun vingers aan de wond zitten, waardoor goede genezing belemmerd kan worden. In toekomstige onderzoek dient het gebruik van een ander proefdiermodel, zoals geiten of schapen, en een extraorale chirurgische benadering te worden overwogen. Net zoals bij tandimplantaten moet ook voor toekomstige experimenten een genezingsperiode voor osseo-integratie worden overwogen voordat het cementloze implantaat wordt ingebracht. Derhalve dient onderzocht te worden of het mogelijk is dat het stevig in elkaar verankeren van de modules kan worden uitgesteld tot een tweede operatie verscheidene maanden later. Zo zullen de stelen van het implantaat niet worden

belast tijdens de initiële genezingsperiode. Ook de zoektocht naar een ideale aanpassing van het oppervlak of coating om de weke delen beter aan het metalen oppervlak te laten hechten, moet verder gaan.

Weefsel engineering methoden om eigen bot van de patiënt te kweken, zullen ongetwijfeld de ideale oplossing zijn voor botreconstructie. Ook al zijn er heel wat succesvolle verslagen van botengineering beschikbaar in *in vitro* en kleine proefdiermodellen, toch zijn er maar weinig verslagen van klinisch succes. De minder positieve resultaten bij de klinische gevallen zijn te wijten aan verschillende factoren, waaronder een onvoldoende vascularisatie van het implantaat en het gekweekte weefsel, een gecompromitteerd zacht weefsel bed en biomechanische factoren.

In dit proefschrift werd botweefselengineering getest voor de regeneratie van een klein alveolair defect en van een groot mandibulair segmentaal defect dat onderhevig was aan functionele belasting. Uit deze onderzoeken blijkt dat de PCL scaffold, ingeplant met TCP of bedekt met een CHA laag, niet osteoinductief is. Het ideale scaffold materiaal zou een resorptietijd moeten hebben die overeenstemt met de snelheid van nieuwe botingroei. Aangezien de PCL scaffold mogelijk te veel tijd nodig heeft om te degraderen, wordt deze door het lichaam gezien als een vreemd materiaal, wat aanleiding geeft tot fibreuze afkapseling. Daarom moet er onderzoek worden uitgevoerd om de PCL scaffold zodanig aan te passen dat er een optimalere resorptietijd ontstaat.

Voor mandibulaire reconstructie bleek de PCL scaffold in onvoldoende mate belasting te kunnen weerstaan. Er was een hoog infectiepercentage en ook kwamen de fixatieplaten en de PCL endoprothese kwamen los. Niettemin presteerden de PCL scaffolds met BMP-2 beter wat betreft botvorming en mechanische testen dan lege PCL scaffolds en scaffolds geladen met autologe BMSC's. Toch zijn er steeds meer verslagen, zoals bijv. Perri *et al.*, 2007, Shah *et al.*, 2008 en Carragee *et al.*, 2013, die beschrijven dat het klinische gebruik van BMP-2 voor botregeneratie geassocieerd wordt met een enorme zwelling van de weke delen na de operatie, ectopische botvorming en mogelijke oncogene effecten. Deze complicaties en ook de hoge kostprijs kunnen het routinematige klinische gebruik hiervan dan ook in de weg staan. Momenteel heeft het Centre for Devices and Radiological Health (CDRH) van de Amerikaanse Food and Drug Administration (FDA) rhBMP-2 nog niet goedgekeurd voor gebruik bij mandibulaire continuïteitsdefecten en daarom dient deze toepassing te worden beschouwd als 'off-label'.

Er moet dus verder onderzoek worden uitgevoerd zodat botweefsel engineering op klinisch vlak nog meer succes behaalt. Voor een groot defect dat een mechanische

belasting moet aankunnen zoals de mandibula moet er bijzondere aandacht worden besteed aan de evaluatie van de complexe biomechanische krachten. Een microvasculaire anastomose kan nog steeds nodig zijn in combinatie met botweefsel engineering methodes om een voldoende vascularisatie van de reconstructie te bereiken.

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LIST OF PUBLICATIONS
CURRICULUM VITAE

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List of Publications

These PhD theses are based on the following original publications:

Chanchareonsook N, Tideman H, Feinberg SE, Hollister SJ, Jongpaiboonkit L, Kin L, Jansen JA. Subcutaneous tissue response to titanium, poly(ϵ -caprolactone), and carbonate-substituted hydroxyapatite-coated poly(ϵ -caprolactone) plates: a rabbit study. *Journal of Biomedical Materials Research: Part A*. 2013 Aug; 101(8):2258-66. doi: 10.1002/jbm.a.34542. Epub 2013 Jan 24. PubMed PMID: 23349120.

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Curriculum Vitae



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